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16. ABSTRACT

Chloroform is ubiquitous in the environment. The northern hemisphere background average has been determined to be approximately 14 ppt and the global average is 8 ppt. After inhalation or ingestion, highest concentrations are found in tissues with high lipid content. Chloroform has been detected in fetal liver. Chloroform would be expected to appear in human milk, because it has been found in cow's milk, cheese, and butter. Subchronic and chronic toxicity experiments with rats, mice, and dogs did not clearly establish a no-effect exposure level for systemic toxicity. Chloroform appears to have teratogenic potential when inhaled. Delayed fetal development occurred at an exposure level (30 ppm) that produced minimal maternal effects. The majority of the assays for mutagenicity and genotoxicity have yielded negative results; however, many of these results are inconclusive because of various inadequacies in the experimental protocols used. The evidence for carcinogenic activity of chloroform is based on increased incidence of hepatocellular carcinomas in male and female B6C3F1 mice, renal epithelial tumors in male Osborne-Mendel rats, kidney tumors in male ICI mice, and hepatomas in NLC and female Strain A mice. Based on International Agency for Research on Cancer criteria for animal studies, this level of evidence would be sufficient for concluding that chloroform is carcinogenic in experimental animals. Available epidemiological data for human carcinogenicity of chloroform are inadequate.

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PREFACE

The Office of Health and Environmental Assessment has prepared this health assessment to serve as a "source document" for EPA use. This health assessment document was developed for use by the Office of Air Quality Planning and Standards to support decision-making regarding possible regulation of chloroform as a hazardous air pollutant. However, the scope of this document has since been expanded to address multimedia aspects.

In the development of the assessment document, the scientific literature has been inventoried, key studies have been evaluated and summary/conclusions have been prepared in order to quantitatively identify the toxicity of chloroform and related characteristics. Observed effect levels and other measures of dose-response relationships are discussed, where appropriate, to place the nature of the health responses in perspective with observed environmental levels.

Any information regarding sources, emissions, ambient air concentrations, and public exposure has been included only to give the reader a preliminary indication of the potential presence of this substance in the ambient air. While the available information is presented as accurately as possible, it is acknowledged to be limited and dependent in many instances on assumption rather than specific data. This information is not intended, nor should it be used, to support any conclusions regarding risks to public health.

If a review of the health information indicates that the Agency should consider regulatory action for this substance, a considerable effort will be undertaken to obtain appropriate information regarding sources, emissions, and ambient air concentrations. Such data will provide additional information for drawing regulatory conclusions regarding the extent and significance of public exposure to this substance.

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1. SUMMARY AND CONCLUSIONS

1.1 INTRODUCTION

Chloroform is a dense, colorless, volatile liquid used primarily in the production of chlorodifluoromethane (90%) and for export (5%). Non-consumptive uses (5%) include use as a solvent, as a cleaning agent, and as a fumigant ingredient. Although chloroform production and capacity have declined recently, 1981 data place direct United States production of chloroform at 184 million kg, with indirect production estimated at 13.2 million kg ($\simeq$193 million kg overall). Also, based on 1981 data, the amount of chloroform emitted to air is estimated to be 7.2 million kg, with emissions to water of 2.6 million kg, and emissions to land of 0.6 million kg. Total United States emissions are estimated (1981) to be 10.4 million kg.

Chloroform is ubiquitous in the environment, having been found in urban and non-urban locations. There have been reports of a northern hemisphere background average of 14 ppt (10^{-12} v/v), with an average in the southern hemisphere of <5 ppt, and a global average of 8 ppt. However, a more recent report suggests the ratio of hemispheric concentrations (north v. south) may be less dramatic, more on the order of 1.6. This same research also suggests that chloroform in the atmosphere may, on a global basis, be largely natural in origin (from tropical oceans, in particular), rather than mostly anthropogenic as previously thought. For the most part, urban ambient air concentrations remain <1000 ppt, and in rural or remote locations concentrations can be <10 ppt. There are some notable exceptions, however, but the reasons for them are not readily apparent. The highest values reported were in Rutherford, New Jersey (31,000 ppt), and Niagara Falls, New York (21,611 ppt).

1.2 PHYSICAL AND CHEMICAL PROPERTIES, AND ANALYSIS

Hydroxyl radical oxidation is the primary atmospheric reaction of chloroform. Based on the rate constant for reaction with chloroform, a half-life of 11.5 weeks is expected. The principal products from this reaction are HCl and CO_2. It has been estimated that roughly 1% of the tropospheric chloroform will diffuse into the stratosphere, based on a lifetime of 0.2 to 0.3 year and a troposphere-to-stratosphere turnover time of 30 years. An EXAMS model of chloroform in water confirms other data suggesting that the major removal process for chloroform in water is evaporation.

The best analytical method for detection of chloroform appears to be gas chromatography with electron capture or electrolytic conductivity detection. This gives a detection limit of <5 ppt.

1.3 PHARMACOKINETICS

The pharmacokinetics and metabolism of chloroform have been studied in both humans and experimental animals. Chloroform is rapidly and extensively absorbed through the respiratory and gastrointestinal tracts. Absorption through the skin would make a significant contribution to body burden only in instances of contact of the skin with liquid chloroform.

The limited available data suggest that, in a human at rest, at least 2 hours are required to reach an apparent equilibrium of the body with the inhaled chloroform concentration. The magnitude of chloroform uptake into the body (dose or body burden) is directly proportional to the concentration of chloroform in the inspired air, the duration of exposure, and the respiratory minute volume.

The absorption of chloroform from the gastrointestinal tract appears to be virtually complete, judging from recovery of unchanged chloroform and metabolites in the exhaled air of humans and in the exhaled air, urine, feces, and carcasses of experimental animals. Chloroform given in a corn oil vehicle to experimental animals is absorbed more slowly than chloroform given in water. Peak blood levels occurred at ≃1 hour after oral administration of chloroform in olive oil to humans or animals.

After inhalation or ingestion, highest concentrations of chloroform are found in tissues with higher lipid contents. Results from the administration of ^{14}C-labeled chloroform to animals indicate that the distribution of radioactivity (reflecting both chloroform and its metabolites) may be affected by the route of exposure. Oral administration appeared to result in the accumulation of a greater proportion of radioactivity in the liver than did inhalation exposure, but differences in experimental protocols make this interpretation tentative. Differences in the distribution of chloroform and its metabolites between male and female animals were found only in mice and not in rats or squirrel monkeys. The kidneys of male mice accumulated strikingly more radioactivity than did those of female mice.

Chloroform has been detected in fetal liver. Chloroform would be expected to appear in human milk, because it has been found in cow's milk, cheese, and butter.

Chloroform is metabolized via microsomal cytochrome P-450 oxidation to trichloromethanol, which spontaneously dehydrochlorinates to the toxic reactive intermediate compound, phosgene. The end products of the phosgene reaction with cellular water are CO_2 and hydrochloric acid, but significant amounts of phosgene and other reactive intermediates bind covalently to tissue macromolecules or conjugate with cysteine and glutathione. Covalent binding of the reactive intermediates to macromolecules is considered to be responsible for the hepato- and nephrotoxicity of chloroform. While the liver is the primary site for chloroform metabolism, other tissues, including the kidney, can also metabolize chloroform to CO_2.

There is no evidence to suggest any qualitative difference for chloroform metabolic pathways in mice, rats, and humans. Interspecies comparisons of the magnitude of chloroform metabolism have been made only for the oral route. Metabolism of chloroform across species, including mice, rats, squirrel monkeys, and humans is proportional to the surface area of the species. The end metabolite, CO_2, is excreted in expired air. Dose dependent pulmonary exhalation is the principal route of excretion for unmetabolized chloroform. Small amounts of chloroform metabolites are excreted in the urine and feces. Results from observations in humans suggest that chloroform metabolism is rate limited.

Regardless of the route of entry into the body, chloroform is excreted unchanged through the lungs and eliminated via metabolism, with the primary stable metabolite, CO_2, also being excreted through the lungs. High concentrations of unchanged chloroform have been found in the bile of squirrel monkeys after oral administration, but not in the urine or feces. The inorganic chloride generated from chloroform metabolism is excreted via the urine.

Decay curves for the pulmonary excretion of unchanged chloroform in humans appear to consist of three exponential components. The terminal component, thought to correspond to elimination from adipose tissue, had a half-time of 36 hours. This long half-time (36 hr) of chloroform residence in the human fat compartment indicates that fatty tissue concentrations of chloroform will not achieve steady-state equilibrium conditions with exposure concentrations until 6 to 7 days of continuous exposure to ambient concentrations, or longer for repetitive daily exposures in the workplace. Conversely, the long residence time of chloroform in the fat compartments of humans indicates that complete desorption of chloroform from these compartments requires 6 to 7 days in chloroform-free environs.

1.4 HEALTH EFFECTS OVERVIEW

Neurological, hepatic, renal, and cardiac effects have been associated with exposure to chloroform. These effects have been documented in humans as well as in experimental animals. In addition, studies with animals indicate that chloroform is carcinogenic and may be teratogenic.

1.4.1 Toxicity

Evidence of chloroform's effects on humans has been obtained primarily during the use of this chemical as an inhalation anesthetic. In addition to depression of the central nervous system, chloroform anesthesia was associated with cardiac arrhythmias (and some cases of cardiac arrest), hepatic necrosis and fatty degeneration, polyuria, albuminuria, and in cases of severe poisoning, renal tubular necrosis. When used for obstetrical anesthesia, chloroform was likely to produce respiratory depression in the infant. Experimental exposures of humans to chloroform have focused only on subjective responses. Humans exposed experimentally to chloroform for 20 to 30 minutes have reported dizziness, headache, giddiness, and tiredness at concentrations >1000 ppm, and light intoxication at concentrations above 4000 ppm.

Similar symptoms occurred in workers employed in the manufacture of lozenges containing chloroform; exposure concentrations ranged from 20 to 237 ppm, with occasional brief exposure to ≈1000 ppm. Additional complaints were of gastrointestinal distress, and frequent and scalding urination. The only other report of adverse effects stemming from occupational exposure to chloroform was of enlargement of the liver; this report was compromised by the apparent lack of suitable controls.

Acute inhalation experiments with animals revealed that single exposures to 100 ppm were sufficient to produce mild hepatic effects in mice. The exposure level that would produce mild renal effects is not known, but frank toxic effects occurred in the kidneys of male mice exposed to 5 mg/L (1025 ppm). In subchronic inhalation experiments, histological evidence of mild hepato- and nephrotoxicity occurred in rats with exposures to as low as 25 ppm, 7 hours/day for 6 months. The effects were reversible if exposure was terminated, and did not occur when exposure was limited to 4 hours/day.

Information on the effects of acute and long-term oral exposure to chloroform is available primarily from experiments with animals. Human data are mainly in the form of case reports and involve the abuse of medications containing not only chloroform, but other potentially toxic ingredients as well; however, a fatal dose of as little as 1/3 ounce was reported. As with inhalation exposure, the primary effects of oral exposure were hepatic and renal damage. Narcosis also occurred with high doses, but this effect was not usually a focus of concern in these experiments. Subchronic and chronic toxicity experiments with rats, mice, and dogs did not clearly establish a no-effect level of exposure for systemic toxicity. Although a dose level of 17 mg/kg/day of chloroform produced no adverse effect in four strains of mice, the lowest dosage tested, 15 mg/kg/day, elevated some clinical chemistry indices of hepatic damage in dogs and appeared to affect a component of the reticuloendothelial system (histiocytes) in their livers.

No controlled studies have been performed to define dose-response thresholds for neurological or cardiac effects of ingested or inhaled chloroform. It is not known whether subtle impairment of neurological or cardiac function might occur at levels as low as or lower than those which affect the liver.

Several substances that are of interest because of accidental or intentional human exposure have been shown to modify the systemic toxicity of chloroform, usually by modifying the metabolism of chloroform to the reactive intermediate. Examples of substances that potentiate chloroform-induced toxicity are ethanol, PBBs, ketones and steroids. Factors that appear to protect against toxicity include disulfiram and high carbohydrate diets.

1.4.2 Reproductive Effects

Observations reported in four articles and two abstracts indicate that chloroform at the concentrations used in these studies has the potential for causing adverse effects in pregnancy maintenance, delays in fetal development, and the production of terata in laboratory animals. The studies which administered chloroform by inhalation 7 hr/day reported more severe outcomes than other studies that administered chloroform by intubation, once or twice a day. The adverse effects produced in the conceptus were observed in association with maternal toxicity, however, the type and severity of effects appeared to be specific to the conceptus, affecting development to a much greater degree than the occurrence of maternal toxicity. It is concluded that chloroform is a potential developmental toxicant. The results of a preliminary study indicate that chloroform has no significant adverse behavioral effect on the fetus or produces embryotoxic effects only at maternally toxic levels.

1.4.3 Mutagenicity

It has been demonstrated that chloroform can be metabolized *in vivo* and *in vitro* to a substance (presumably phosgene) that interacts with protein and lipid. The potential for metabolically activated chloroform to bind to DNA cannot be determined from the available studies, but, if binding to DNA does occur, it would be at a very low level.

The majority of the assays for genotoxicity have yielded negative results. However, many of these results are inconclusive because of inadequacies in the experimental protocols. The major problem has been the use of exogenous activation systems (i.e., S9 mix). In none of the studies was it shown that chloroform was activated or metabolized by the activation system used. Metabolism of 2-aminoanthracene or vinyl compounds (used as positive controls) is an inadequate indication that the activation system can metabolize chloroform because these substances are not halogenated alkanes. A better indication that the activation system is sufficient for metabolism of chloroform may be to show that it metabolizes $^{14}CHCl_3$ to intermediates that bind to macromolecules. A second problem in the use of exogenous activation systems is the possibility that highly reactive metabolites may react with microsomal or membrane lipid or protein before reaching the DNA of the test organism. Another problem in *in vitro* tests is that adequate precautions are sometimes not taken to prevent the escape of volatilized chloroform.

On the basis of presently available data, no definitive conclusion can be reached concerning the mutagenicity of chloroform. However, evidence from studies measuring binding to macromolecules, DNA damage, and mitotic arrest suggest that chloroform may be mutagenic. Alternatively, because recent studies on the mechanism of action of tumor promoters suggest that promoters can damage DNA, chloroform may promote carcinogenesis rather than initiate it.

1.4.4 Carcinogenicity

The carcinogenic potential of chloroform has been experimentally evaluated in several animal species and by epidemiologic surveys. Chronic animal studies have been conducted in eight strains of mice, two strains of rats, and beagle dogs. In all of these studies chloroform was administered by the oral route and not by inhalation, an important route of chloroform exposure for humans. However, a carcinogenic response from chloroform exposure is not expected to be dependent upon the route of assimilation into the body.

Evidence for the carcinogenicity of chloroform in experimental animals includes: statistically significant increases in renal epithelial tumors in male Osborne-Mendel rats (two studies); hepatocellular carcinomas in male and female $B_6C_3F_1$ mice; kidney tumors in male ICI mice; and hepatomas in female Strain A mice and NLC mice. Chloroform has also been shown to promote growth and metastasis of murine tumors. In these cancer studies the carcinogenicity of chloroform is organ specific, primarily liver and kidney, the target organs of acute chloroform toxicity and covalent binding as well.

The carcinogenicity of chloroform was first investigated in 1945. Although the number of animals in each test group was small and the mortality was high at the higher doses, an increased incidence of hepatomas was observed in the Strain A mice. Induction of hepatomas was confirmed in 1967 in a very limited study in NLC mice. Chloroform was administered in oil by gavage in both studies.

In 1976, male and female $B_6C_3F_1$ mice, chloroform-treated by corn oil gavage, showed highly significant dose-dependent increases in hepatocellular carcinomas, with metastases to the lungs in some mice. In a similar study, statistically significant dose-dependent increases of kidney epithelial tumors were found in male Osborne-Mendel rats.

In another study (1979), kidney tumors were observed in male ICI mice administered chloroform in either toothpaste or arachis oil vehicle.

In the most recently published study (1985), chloroform administered in the drinking water of male Osborne-Mendel rats induced a statistically significant increase in the incidence of renal tumors, thus supporting the findings from the earlier study in which chloroform was administered in corn oil by gavage. Female $B_6C_3F_1$ mice, however, did not show an increase in the incidence of liver tumors when chloroform was administered in the drinking water. This was inconsistent with the positive findings reported in previous investigations of chloroform oil gavage treatment of mice. The lack of response of the mice in the drinking water study versus the highly significant response of these mice when chloroform was given in corn oil vehicle as a single bolus, suggests that chloroform-induced heptatocellular carcinomas in this strain of mice may be related to absorption patterns, the dosing regimen, peak blood levels of chloroform, and target tissue levels of its reactive intermediate metabolites. The corn oil carrier has not been shown to induce an increase in the incidence of liver tumors in mice.

Other studies of chloroform carcinogenicity have shown negative results. Treatment with a gavage dose of chloroform in toothpaste did not produce a carcinogenic response in female ICI mice or in male mice of the CBA, C57BL, and CF/1 strains, nor was a carcinogenic response observed in male or female Sprague-Dawley rats given chloroform in toothpaste by gavage, but early mortality was high in control and treatment groups. Gavage doses of chloroform in toothpaste did not cause a carcinogenic response in male and female beagle dogs treated for over 7 years, although there was an increased incidence of hepatic nodular hyperplasia. The daily chloroform doses given to mice and rats in toothpaste or arachis oil were lower than those given in corn oil or drinking water in studies showing a positive carcinogenic response. In newborn (C57 x DBA2-F1) mice given subcutaneous doses during the initial 8 days of life and observed for their lifetimes, a carcinogenic effect of chloroform was not evident. The dose levels used appeared well below a maximum tolerated dose and the period of treatment was quite short. In Strain A mice, chloroform was ineffective at maximally tolerated and lower doses in a pulmonary adenoma bioassay. However, other chemicals that have shown carcinogenic activity in different tests were ineffective in this particular Strain A mouse pulmonary adenoma bioassay. Chloroform does not induce transformation of Syrian baby hamster kidney cells (BHK-21/Cl 13) *in vitro*.

While no epidemiological studies have evaluated chloroform by itself, several studies have been made of populations with chlorinated drinking water, in which chloroform is the predominant chlorinated hydrocarbon compound. Small increases in rectal, bladder, and colon cancer were consistently observed by several case-control and ecological studies, several of which are statistically significant. Because other possible carcinogens were present along with chloroform, it is impossible to identify chloroform as the sole carcinogenic agent. Therefore, the epidemiologic evidence for chloroform's carcinogenicity must be termed inadequate.

It is generally accepted that the carcinogenic activity of chloroform resides in its highly reactive intermediate metabolites such as phosgene. Irreversible binding of chloroform metabolites to cellular macromolecules supports several theoretical concepts of the mechanism(s) for its carcinogenicity. Available data on chloroform metabolism and pharmacokinetics pertinent to the conditions of the carcinogenicity bioassays are used in the extrapolation of the dose-carcinogenic response relationships of laboratory animals to humans. There is no difference in absorption of chloroform across species. Also, there is no evidence to suggest any qualitative difference in the metabolic pathways or metabolite profiles of mice, rats, and humans for chloroform. An experimental basis exists for determining relative amounts of chloroform metabolized in various species, including man, and this information has been used in the risk assessment for chloroform.

The quantitative risk assessment is based on the assumption of a non-threshold mechanism, and consequently mathematical extrapolation models consistent with this assumption were evaluated. Although the nonthreshold mathematical risk extrapolation model is conservative based upon a public health point of view, the correction used in the calculation of a human equivalent dose is scientifically conservative and may lead to an overestimate of the amount of chloroform metabolized in the test animals, and hence underestimate the risk. In addition, experimental data that include covalent binding in human tissues suggest that humans may have a greater than expected capacity to metabolize chloroform when compared to rodents, again indicating the possibility of a higher risk for humans than estimated in the assessment. Using the linearized multistage model, the geometric mean, 8.1×10^{-2} per mg/kg/day, of the slope estimates, q_1^*, calculated from chloroform-induced liver tumors in male and female mice treated by gavage, is the value used to compare the relative potency of chloroform to other carcinogens and to calculate the unit risk for drinking water and air. The upper-bound estimate of cancer risk due to 1 μg/L of chloroform in water is consistent with the limited epidemiologic data available for humans.

Five data sets are used to estimate the carcinogenic risk of chloroform. The end points include liver tumors in female mice (NCI, 1976), liver tumors in male mice (NCI, 1976), kidney tumors in male rats (NCI, 1976, and Jorgenson et al., 1985) and kidney tumors in male mice (Roe et al., 1979). The unit risk values at 1 mg/kg/day, calculated by the linearized multistage model on the basis of these data sets, are comparable. The risk value is useful for estimating the possible magnitude of the public health impact. The upper-bound incremental cancer risk derived from the geometric mean of 4 data sets, chloroform gavage studies which showed a statistically significant increase of hepatocellular carcinomas in mice, is 8.1×10^{-2} per mg/kg/day. The CAG potency index for chloroform (defined as the slope x molecular weight) is 1×10^{1}, ranking it in the lowest quartile of 55 chemicals that the CAG has evaluated as suspect carcinogens. The upper-bound estimate of the incremental cancer risk due to ingesting 1 µg/L of chloroform in drinking water is 2.3×10^{-6}. The upper-bound estimate of the incremental cancer risk due to inhaling 1 $\mu g/m^3$ of chloroform in air based upon positive gavage carcinogenicity studies is 2.3×10^{-5}. The upper-bound nature of these estimates is such that the true risk is not likely to exceed this value and may be lower.

Based on EPA's proposed Carcinogen Risk Assessment Guidelines, chloroform is classified as having sufficient animal evidence and inadequate epidemiologic evidence. The overall weight-of-evidence classification is group B2, meaning that chloroform is probably carcinogenic in humans. Applying the International Agency for Research on Cancer (IARC) criteria, the level of animal evidence for carcinogenicity is sufficient, and the overall

IARC classification is group 2B, meaning that chloroform should be considered to be a probable human carcinogen.

2. INTRODUCTION

The U.S. Environmental Protection Agency is responsible under the authority of various laws for the identification, comprehensive assessment, and as appropriate regulation, of environmental substances which may be of concern to the public health. For example, under Section 112 of the Clean Air Act the EPA Administrator is directed to establish standards for any air pollutant (other than those for which national ambient air quality standards are applicable) which, in his judgment, "causes, or contributes to, air pollution which may reasonably be anticipated to result in an increase in mortality or an increase in serious irreversible, or incapacitating reversible, illness."

Within EPA, the Office of Health and Environmental Assessment is responsible for providing scientific assessments of health effects for potentially hazardous air pollutants such as chloroform. These health assessment documents form the scientific basis for subsequent agency actions, including the Administrator's judgment as to whether regulations or standards may be appropriate.

This Health Assessment Document for Chloroform represents a comprehensive data base that considers all sources of chloroform in the environment, the likelihood for human exposures, and the possible consequences to man and lower organisms from its absorption. This information is integrated into a format that can serve as the basis for qualitative and quantitative risk assessments, while at the same time identifying gaps in our knowledge that limit present evaluative capabilities. Accordingly, it is expected that this document may serve the information needs of many government agencies and private groups that may be involved in decision making and regulatory activities. (As with all such EPA documents, a preliminary draft was made available to the scientific community and the general public so that comments of interested individuals and organizations could be considered, and the latest scientific evidence incorporated, in the final draft. The preliminary draft was also reviewed by the Environmental Health Advisory Committee of EPA's Science Advisory Board at a public meeting (49 Federal Register 9609).

3. BACKGROUND INFORMATION

3.1. INTRODUCTION

This section provides background information supportive of the human health effects data presented in subsequent sections. It is not intended to be a comprehensive review of analytical methodology, sources, emissions, air concentrations, or environmental transport and fate information. In order to fulfill this purpose and since the literature concerning chloroform is vast, only a portion of the available literature was included. Those articles included were chosen because of their relevance to the topic at hand and because they were representative of the literature as a whole.

To provide the most complete overview possible, some non-peer reviewed information has been added, from the Chloroform Materials Balance Draft Report by Rehm et al. (1982). This is an updated version of the original Level I Materials Balance: Chloroform (Wagner et al., 1980). As described by Wagner et al. (1980):

> A Level I Materials Balance requires the lowest level of effort and involves a survey of readily available information for constructing the materials balance. Ordinarily, many assumptions must be made in accounting for gaps in information; however, all are substantiated to the greatest degree possible. Where possible, the uncertainties in numerical values are given, otherwise they are estimated. Data gaps are identified and recommendations are made for filling them. A Level I Materials Balance relies heavily on the EPA's Chemical Information Division (CID) as a source of data and references involving readily available information. Most Level I Materials Balance are completed within a 3-6 week period; CID literature searches generally require a 2 week period to complete. Thus, the total time required for completion of a Level I materials balance ranges from 5-7 weeks.

Because a greater level of effort went into the 1980 and 1982 Materials Balance reports on chloroform than would normally be devoted to background (non-health effects) information in an EPA health assessment document, information in this chapter is drawn from these reports. However, because such information has not been peer-reviewed and includes some major assumptions, it should not be used to support any regulations or standards regarding risks to public health.

3.2. PHYSICAL AND CHEMICAL PROPERTIES

Chloroform ($CHCl_3$) (CAS registry number 67-66-3) is a member of a family of halogenated saturated aliphatic compounds. Synonyms for chloroform include the following:

Chloroforme (French)
Chloroformio (Italian)
Formyl trichloride
Methane trichloride
Methane, trichloror
Methenyl chloride
Methenyl trichloride
Methyl trichloride
NCI-C02686
Trichloromethaan (Dutch)
Trichloroform
Trichloromethane

Table 3-1 lists various physical properties. Chloroform is a colorless, clear, dense, volatile liquid with an ethereal non-irritating odor (DeShon, 1979). Chloroform is nonflammable; however, when hot chloroform vapors are mixed with alcohol vapors, the mixture burns with a greenish flame. At 25°C and 1 atmosphere, a 1 ppm concentration of chloroform in air is equal to 4.88 mg/m^3.

Chloroform decomposes with prolonged exposure to sunlight regardless of the presence of air (DeShon, 1979). It also decomposes in the dark in the presence of air. The principal decomposition products include phosgene, hydrogen chloride, chlorine, carbon dioxide, and water. Ozone causes chloroform to decompose rapidly.

Chloroform forms a hydrate in water at 0°C ($CHCl_3 \cdot 18H_2O$, CAS registry number 67922-19-4); the hexagonal crystal decomposes at 1.6°C (DeShon, 1979).

TABLE 3-1. PHYSICAL PROPERTIES OF CHLOROFORM

Molecular weight	119.38
Melting point (°C)	-63.2
Boiling point (°C)	61.3
Water-chloroform azeotrope (°C)	56.1
Specific gravity (25/4°C)	1.48069
Vapor density (101 kPa, 0°C, kg/m^3)	4.36

Vapor pressure

°C	kPa	torr
-30	1.33	10.0
-20	2.61	19.6
-10	4.63	34.7
0	8.13	61.0
10	13.40	100.5
20	21.28	159.6
30	32.80	246.0
40	48.85	366.4

Solubility in water

°C	g/kg H_2O
0	10.62
10	8.95
20	8.22
30	7.76

Log octanol/water partition coefficient: 1.97[a]

Conversion factors at 25°C and 1 atm[b]

1 ppm $CHCl_3$ in air equals 4.88 mg/m^3

1 mg/m^3 $CHCl_3$ in air equals 0.205 ppm

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[a] Hansch and Leo, 1979.

[b] Calculated.

SOURCE: DeShon, 1979.

Chloroform is chemically stable to water, having a hydrolysis half-life of 3100 years in neutral water at 25°C (Mabey and Mill, 1978); the half-life of chloroform in air from hydroxyl radical reactions is 78 days (Hampson, 1980).

The hydrogen atom in chloroform can be removed in the presence of warm alkali metal hydroxide to form a trichloromethyl anion (DeShon, 1979). This anion can condense with carbonyl compounds. Both wet and dry chloroform will react with aluminum, zinc, and iron.

Small amounts of ethanol are used to stabilize chloroform from oxidation during storage (DeShon, 1979).

3.3. SAMPLING AND ANALYSIS

3.3.1. Chloroform in Air

Chloroform in air can be analyzed by a number of methods; however, the method of Singh et al. (1980) appears to be substantially free of artifact problems and completely quantitative. In this method, an air sample in a stainless steel canister at 32 psig is connected to a preconcentration trap consisting of a 4" x 1/16" ID stainless steel tube containing glass beads, glass wool, or 3% SE-30 on acid washed 100/120 mesh chromosorb W. The sampling line and trap, maintained at 90°C, are flushed with air from the canister; then the trap is immersed in liquid O_2 and air is passed through the trap, the initial and final pressure being noted (usually between 30 and 20 psig) on a high-precision pressure gauge. The ideal gas law can be used to estimate the volume of air passed through the trap. The contents of the trap are desorbed onto a chromatography column by backflushing it with an inert gas while holding the trap at boiling water temperature. An Ascarite trap may be inserted before the chromatography column to remove water. Suitable columns include 20% SP-2100 and 0.1% CW-1500 on Supelcoport (100/120 mesh, 6' x 1/8" stainless steel) and 20% DC-200 on Supelcoport (80/100 mesh, 33' x 1/8" Ni). Both columns can be operated at 45°C with a carrier gas flow of 40 ml/min on the former column and 25 ml/min on the latter. An electron capture detector operating at 325°C was found to be optimum. It should be noted that the above authors found Tenax to be unsuitable for air analyses because of the presence of artifacts in the spectrum from oxidation of the Tenax monomer. In addition, when Tenax is used as a sorbent, safe sampling volumes (i.e., that volume of air which, if sampled over a variety of circumstances, will not cause significant breakthrough) should be adhered to. Brown and Purnell (1979) determined the safe volume for chloroform per gram Tenax to be 9.3 L (flow rate 5-600 ml/min; $CHCl_3$ conc. <250 mg/m^3; temp. up to 20°C) with a safe desorption temperature of 90°C.

The detection limits of this method were not specified and are dependent on the volume of air sampled. Analyses as low as 16 ppt have been reported using this method (Singh et al., 1980).

3.3.2. Chloroform in Water

Chloroform in water can be analyzed by the purge-and-trap method (Method 502.1) as recommended by the Environmental Monitoring and Support Laboratory of the U.S. EPA (1981a). In this method, an inert gas is bubbled through 5 ml of water at a rate of 40 ml/minute for 11 minutes, allowing the purgeable organic compounds to partition into the gas. The gas is passed through a column containing Tenax GC at 22°C, which traps most of the organics removed from the water. The Tenax column is then heated rapidly to 130°C and backflushed with helium (20-60 ml/minute, 4 minutes) to desorb the trapped organics. The effluent of the Tenax column is passed into an analytical gas chromatography column packed with 1% SP-1000 on Carbopack-B (60/80 mesh, 8'x 0.1" ID) maintained at 40°C. The column is then temperature programmed starting at 45°C for 3 minutes and increasing at 8°C/minute until

220°C is reached; it is then held there for 15 minutes or until all compounds have eluted. A halogen-specific detector (or GC-MS) having a sensitivity of 0.10 μg/L with a relative standard deviation of <10% must be used.

3.3.3 Chloroform in Blood

Chloroform in blood can be analyzed by using a modified purge-and-trap method (Pellizzari et al., 1979). This method involves diluting an aliquot of whole blood (with anticoagulant) to ≃50 ml with prepurged, distilled water. The mixture is placed in a 100 ml 3-neck round bottom flask along with a Teflon-lined magnetic stirring bar. The necks of the flask are equipped with a helium inlet, a Tenax trap, and a thermometer. The Tenax trap is a 10 cm x 1.5 cm ID glass tube containing pre-extracted (Soxhlet, methanol, 24 hr) and conditioned (270°C, 30 ml/min helium flow, 20 min) 35/60 mesh Tenax (≃1.6 g, 6 cm). The sample is then heated to 50°C and purged with a helium flow rate of 25 ml/min for 90 min. Analysis can be performed as indicated in Section 3.3.2.

3.3.4. Chloroform in Urine

Chloroform in urine can be analyzed by using an apparatus identical to the one described in Section 3.3.3, using 25 ml of urine diluted to 50 ml instead of blood.

3.3.5. Chloroform in Tissue

Chloroform in tissue can be analyzed by using an apparatus identical to the one described in Section 3.3.3, using 5 g of tissue diluted to 50 ml and macerated in an ice bath instead of blood. The purge time is reduced to 30 minutes.

3.4. EMISSIONS FROM PRODUCTION AND USE

3.4.1. Emissions from Production

3.4.1.1. Direct Production--Chloroform is produced commercially in the United States by two methods, chlorination of methane and chlorination of methyl chloride produced from methanol and hydrogen chloride (Wagner et al., 1980; DeShon, 1979). The chemistry is summarized by the following reactions:

Methane Chlorination

$$4CH_4 + 10Cl_2 \longrightarrow CH_3Cl + CH_2Cl_2 + CHCl_3 + CCl_4 + 10HCl$$

Methanol Hydrochlorination

$$HCl + CH_3OH \xrightarrow[280 - 350^\circ C]{\text{Catalyst}} CH_3Cl + H_2O$$

$$3CH_3Cl + 6Cl_2 \longrightarrow CH_2Cl_2 + CHCl_3 + CCl_4 + 6HCl$$

The methanol process has been reported to account for 74% of capacity, with methane accounting for only 26% of capacity (SRI International, 1983). Moreover, the methanol process is believed to account for an ever increasing proportion of capacity.

In the chlorination of methane, natural gas is directly chlorinated in the gas phase with chlorine at 485-510°C (Anthony, 1979). The product mixture contains all chlorinated methanes, which are removed by scrubbing and separated by fractional distillation.

In the second process, gaseous methanol and HCl are combined over a hot catalyst to form methyl chloride (Ahlstrom and Steele, 1979). The methyl chloride is then chlorinated with chlorine to produce CH_2Cl_2, $CHCl_3$, and CCl_4 (DeShon, 1979). The chlorination conditions for both processes can be adjusted to optimize chloroform production.

United States production is carried out by four manufacturers at six sites summarized in Table 3-2. The annual production of chloroform in the United States has risen from 35 million kg (77 million lb) in 1960 (DeShon, 1979) to >184 million kg (405 million lbs) in 1981 (USITC, 1982) with few declines. The production and capacity of the industry has recently declined, however (see Table 3-2.)

3.4.1.1.1. Chloroform emissions from the methane chlorination process. Rem et al. (1982) reported that air emissions could come from process vents, in-process and product storage tanks, liquid waste streams, secondary emissions (handling and disposal of process wastes), and fugitive emissions from leaks in process valves, pumps, compressors and pressure relief valves.

The emission factors they calculated were based on a typical methane chlorination facility as reported by U.S. EPA (1980a) having a total chloromethane capacity of 2 x 10^5 metric tons (441 x 10^6 lb), operating continuously (8760 hr/year), and having a product mix of 20% CH_3Cl, 45% CH_2Cl_2, 25% $CHCl_3$, and 10% CCl_4. The emission factor for the uncontrolled recycle methane inert gas purge vent for the above plant (0.014 kg/metric ton) was calculated from an hourly $CHCl_3$ emission rate of 0.071 kg/hr reported by Dow Chemical Company for a 46,000 metric ton/year facility assuming continuous (8760 hr/year) operation (Letter from J. Beale, n.d., Dow Chemical U.S.A., Midland, Michigan, to L. Evans, Emissions Standards and Engineering Division, U.S. EPA, concerning Dow facility at Freeport, TX; cited in Rehm et al., 1982). The uncontrolled emission factor for the distillation area emergency inert gas vent (0.032 kg/metric ton) was calculated from an emission factor for volatile organic compounds (VOC) of 0.20 kg/metric ton of total chloromethane production and composition data showing chloroform to be 4% of VOC (U.S. EPA, 1980a). In-process and product storage emissions (0.91 to 0.80 kg/metric ton, depending on controls) were calculated from emission equations for breathing and working losses from AP-61 (U.S. EPA, 1981b) assuming tanks to be half-full, have 95% emission

TABLE 3-2. CHLOROFORM PRODUCERS, PRODUCTION SITES, AND CAPACITIES[a]

Producer	Production site	Capacity Millions of kg (Millions of lb)	Process
Diamond Shamrock	Belle, WV	18 (40)	Methanol
Dow Chemical	Freeport, TX	33 (74)	Methanol
	Plaquemine, LA	27 (60)	Methanol
Linden Chemicals and Plastics, Inc.	Moundsville, WV	14 (30)	Methanol
Vulcan Materials Co.	Geismar, LA Wichita, KS	27 (60) 50 (110)	Methanol Methanol and Methane
TOTAL		169 (374)	

[a] One producer, Stauffer Chemical Co., which was included in the SRI study, has been deleted from this table because it reportedly is no longer producing. The capacities and processes for Dow Chemical have also been revised in the table, based on information received from Dow Chemical U.S.A. The totals above reflect these changes.

SOURCE: SRI International, 1983.

controls when present, and a 12°C diurnal temperature variation (U.S. EPA, 1980b). Rehm et al. (1982) calculated the total chloroform emissions to air to be 70.2 metric tons (155 x 10^3 lb) by multiplying the appropriate factors by plant capacity use after including secondary emissions (0.21 kg/metric ton) and fugitive emissions (5.5 kg/hr).

Releases of chloroform to water come from scrubbers, neutralizers, and cooling water (Rem et al., 1982). Based on a 300 ppm $CHCl_3$ content in total wastewater discharges averaging 68 L/min and assuming that 90% would volatilize, Rehm et al. (1982) calculated a release factor of 0.023 kg/metric ton. They then added to this the emission from indirect contact cooling water (100 ppm, 5800 L cooling water/metric ton $CHCl_3$, 90% evaporation) to calculate a release rate of 3.3 metric tons of $CHCl_3$ (7.3×10^3 lb) per year to water.

No quantifiable data were available to Rehm et al. (1982) regarding release of chloroform to land from methane chlorination.

3.4.1.1.2. Chlorination emissions from the methanol hydrochlorination-methyl chloride chlorination process. Chloroform emissions to air come from process vents, in-process and product storage tank emissions, and fugitive emissions from leaks in valves, pumps, compressors, and pressure relief valves (Rem et al., 1982). Rehm et al. (1982) used an uncontrolled emission factor reported by Vulcan Materials Company for process vents (Hobbs, 1978), and assumed continuous (8760 hr/year) operation, and controls sufficient to reduce emissions 80% to obtain the emission factor for controlled process vents (0.003 kg/metric ton for controlled; 0.015 kg/metric ton for uncontrolled). Storage emissions (0.176 kg/metric ton for controlled, 0.88 kg/metric ton for uncontrolled) were calculated from Hobbs (1978) and from emission equations for breathing and working losses from AP-42 (U.S. EPA, 1981b), assuming tanks to be half-full, have 80% emission reduction controls, and a 12°C diurnal temperature variation (U.S. EPA, 1980b). Fugitive emission factors (3.32 kg/hr for uncontrolled; 1.08 kg/hr for controlled) for volatile organic compounds were used (U.S. EPA, 1980c) along with a control factor of 67.5% based on leak detection and repair (U.S. EPA, 1980b). Emission rates were then calculated to be 196 metric tons (432 x 10^3 lb) based on plant capacity, capacity use (66%), the level of emission controls used at each plant, and continuous operation (8760 hr/year).

Rem et al. (1982) assumed that releases of chloroform to water from methyl chloride chlorination resulted from contamination of cooling water and from spent acid and spent caustic streams. For cooling water contamination, they assumed minor spills and leaks resulted in contamination of 100 mg chloroform per liter of cooling water, that 5800 L of cooling water per metric ton of chloroform produced was consumed, and that 90% of the chloroform evaporated. For spent acid and spent caustic streams they assumed that 0.04 kg of chloroform is released for every metric ton of chloromethane produced. They further stated that this release factor was not considered to be very reliable (and in fact industry commenters have suggested that no measurable losses of chloroform are observed under normal operating conditions). However, in the absence of better data Rehm et al. (1982) used this factor to approximate emissions. In addition, they assumed that one third of the chloromethane production consists of chloroform and that 90% of the released chloroform evaporates. Using these assumptions, Rehm et al. (1982) calculated that 0.070 kg of chloroform is released to water per metric ton of chloroform produced, or 8.0 metric tons (17.6 x 10^3 lb) of chloroform were released to water based on 1980 production levels.

Rem et al. (1982) reported that the bottoms from chloroform distillation in the methyl chloride chlorination process are the feed for carbon tetrachloride and perchloroethylene production, and that during their production a residue is formed that contains chloroform. This residue is landfilled or deep-well injected. This represents the only known release of chloroform from carbon-tetrachloride/perchloroethylene production. (Dow

Chemical U.S.A. has, to the contrary, reported that the residue is instead recycled as a raw material feed to production, with no measurable amount of chloroform exiting from the plant.)

Rem et al. (1982) assumed that 1.02 kg of residue is produced per metric ton of chloroform from methyl chloride production (see Wagner et al., 1980). They further assumed (as did Wagner et al., 1980) that 18.4% of the residue is chloroform and that 25% is landfilled (again, these figures have been questioned by Dow Chemical U.S.A.). This results in a release factor of 0.047 kg chloroform per metric ton of chloroform produced, or 5.4 metric tons (12×10^3 lb) based on 1980 production.

3.4.1.1.3. Summary of direct production. Direct production emits some 283 metric tons into the environment on an annual basis. Greater than 95% (266.2 metric tons) of this is emitted into the air. Direct production accounts for ≃3% of all environmentally released chloroform, and ≃3.6% of all chloroform released to air. Table 3-3 summarizes chloroform discharges from direct production.

3.4.1.2. Indirect Production

3.4.1.2.1. Chloroform formation during ethylene dichloride production. Ethylene dichloride (EDC) is produced by two methods, direct chlorination and oxychlorination, and is used principally for vinyl chloride monomer (VCM) production. (See EPA Health Assessment Document for EDC, EPA 600/8-84-006F.) A combination of the two methods is used by most VCM production facilities in a process known as the balanced process since the HCl from the dehydrochlorination of EDC is used to produce more EDC from ethylene, the major products of the overall reaction being VCM and H_2O.

TABLE 3-3. ESTIMATED CHLOROFORM DISCHARGES FROM DIRECT SOURCES

	Environment release (metric tons/year)[a]			
Source	Air	Water	Land	Total
Methyl chloride chlorination	196	8	5.4	209.4
Methane chlorination	70.2	3.3	---	73.5
Total:	266.2	11.3	5.4	282.9

[a] These figures may now be high due to reduced industry capacity, and conversion to the methanol process by all but one production site.

SOURCE: Rehm et al. (1982).

Direct Chlorination

$CH_2 = CH_2 + Cl_2 \longrightarrow CH_2ClCH_2Cl$

Oxychlorination

$2\ HCl + 1/2\ O_2 \xrightarrow{\text{catalyst}} Cl_2 + H_2O$

$CH_2 = CH_2 + Cl_2 \longrightarrow CH_2ClCH_2Cl$

Balanced Process

$CH_2 = CH_2 + Cl_2 \longrightarrow CH_2ClCH_2Cl$

$CH_2 = CH_2 + 2HCl + 1/2\ O_2 \longrightarrow CH_2ClCH_2Cl + H_2O$

$HCl + CH_2 = CHCl \xleftarrow{\Delta}$

Chloroform is formed as a by-product during EDC manufacture. Rehm et al. (1982) estimated chloroform emissions to air from EDC production based on emission factors developed by the EPA during field studies of domestic EDC production facilities (see U.S. EPA, 1980d). Domestic production facilities are listed in Table 3-4. Emission sources (e.g., process vents, fugitive emissions, storage) for each plant, plant capacity, capacity utilization (56%), and control technology were all combined with the emission factors to determine the overall chloroform emissions at the current level of control. According to their calculations, chloroform emissions to the atmosphere are ≃760 metric tons/year (1675 x 103 lb/year).

Chloroform releases to water may occur during the discharge of wastewater from the process; however, the amount of chloroform present is unknown.

Chloroform releases to land from EDC production reportedly occur when the light ends from EDC distillation are landfilled (Rem et al., 1982). An estimated 217 metric tons (478×10^3 lb) were landfilled in 1980. (This estimate may be high; Dow Chemical U.S.A. has commented, for example, that the landfilling of both light and heavy ends from EDC distillation has not occurred since the 1979s.)

3.4.1.2.2. Chlorination of drinking water. Chloroform in drinking water arises when humic substances or methyl ketones (e.g., acetone) in water react with a hypochlorite anion (Stevens et al., 1976; NAS, 1978). Hypochlorite is the principal reactant in chlorinated water above pH 5. Chloroform is produced by the haloform reaction outlined below.

$$R\text{-}COCH_3 + 3OCl^- \longrightarrow RCOCCl_3 + 3OH^-$$

$$R\text{-}COCCl_3 + OH^- \longrightarrow RCOO^- + CHCl_3$$

TABLE 3-4. ETHYLENE DICHLORIDE PRODUCERS, PRODUCTION SITES, AND CAPACITIES

Producer	Production site	Capacity millions of kg	 (millions of lb)
Alantic Richfield Co. ARCO Chem. Co. Div.	Port Arthur, TX	204	(450)
Borden, Inc. Borden Chem. Div. Petrochems. Div.	Geismar, LA	231	(510)
Diamond Shamrock	Deer Park, TX	145	(320)
Dow Chemical. U.S.A.	Freeport, TX	726	(1600)
	Oyster Creek, TX	476	(1050)
	Plaquemine, LA	862	(1900)
E.I. du Pont de Nemours and Co., Inc. Conoco Inc., subsid. Conoco Chems. Co. Div.	Lake Charles, LA	524	(1155)
Ethyl Corp. Chems. Group	Baton Rouge, LA	318	(700)
	Pasadena, TX	102	(225)
Formosa Plastics Corp. U.S.A.	Baton Rouge, LA	249	(550)
	Point Comfort, TX	386	(850)
Georgia-Pacific Corp. Chem. Div.	Plaquemine, LA	748	(1650)
The BF Goodrich Co. BF Goodrich Chem. Group	La Porte, TX	719	(1585)
Convent Chem. Corp., subsid.	Calvert City, KY	454	(1000)
	Convent, LA	363	(800)
PPG Industries, Inc. Chems. Group Chem. Div.	Lake Charles, LA	1225	(2700)

TABLE 3-4. (continued)

Producer	Production site	Capacity millions of kg	(millions of lb)
Shell Chem. Co.	Deer Park, TX	635	(1400)
	Norco, LA[b]	544	(1200)
Union Carbide Corp. Ethylene Oxide Derivatives Div.	Taft, LA[b]	68[a]	(150)
	Texas City, TX[b]	68	(150)
Vulcan Materials Co. Vulcan Chems. Div.	Geismar, LA	159	(350)
	TOTAL	9,206	(20,295)

[a] Captive use only.
[b] Reportedly now shut down.

SOURCE: SRI International, 1983.

Rem et al. (1982) used the data from the National Organics Reconnaissance Survey (NORS) (Symons et al., 1975) and the National Organics Monitoring Survey (NOMS) (U.S. EPA, n.d.) to estimate the concentration of chloroform in drinking water. These surveys provided information on chloroform concentrations in 137 U.S. cities. To determine the amount of chloroform generated, the authors multiplied the volume of water treated by each city by the chloroform concentration in the drinking water. The amount of chloroform generated by each city was then summed and divided by the volume of water generated to give a weighted average concentration of 41 µg/L. This was then multiplied by the estimated volume of water chlorinated annually (4.6 x 10^{13} L/year) to yield the amount in the U.S. (1900 metric tons, 4.2 x 10^{6} lb). Rehm et al. (1982) stressed that this value probably represents a minimum since NORS was conducted during the winter (hence, chloroform levels were low) and NOMS samples were iced when taken (hence, may be lower than if allowed full contact time). Thus, the actual value may be higher than this estimate.

3.4.1.2.3. Chlorination of municipal sewage. Chlorination of municipal sewage results in increased chloroform concentrations (NAS, 1978). Municipal wastewater generally contains a lower concentration of chloroform precursors (humic materials) than do ambient waters; therefore, the amount of chloroform generated from wastewater chlorination is smaller (Wagner et al., 1980). Rehm et al. (1982) calculated chloroform production from wastewater treatment based on analyses of the secondary effluent from 28 municipal plants published by the EPA (U.S. EPA, 1979a). These analyses showed that the average chloroform concentration increased by 9 μg/L, from 5 to 14 μg/L. Rehm et al. (1982) assumed that all municipal wastewater was chlorinated and multiplied the average concentration increase by the municipal wastewater flow (9.7×10^{10} L/day) listed in U.S. EPA (1981c). By this method, 320 metric tons (0.7×10^6 lb) were calculated to be produced annually.

3.4.1.2.4. Chlorination of cooling waters. Cooling water used in electric power generating plants is treated with chlorine as a biocide to prevent fouling intake screens and condensers in both once-through and closed cycle systems (U.S. EPA, 1980e). Rehm et al. (1982) calculated chloroform production based on the size of the average power plant (hence, the volume of water required), the type of cooling system used (once-through or recirculating), and the fact that 65% of all power plants chlorinate cooling water. They calculate that 72 metric tons of chloroform (160×10^3 lb) are discharged directly into water by once-through systems and 190 metric tons of chloroform (420×10^3 lb) are emitted into the air by recirculating systems.

A total of 262 metric tons of chloroform (580 x 10^3 lb) are produced annually from cooling water chlorination.

3.4.1.2.5. Chlorination in the pulp and paper industry. Pulp and paper mills emit more chloroform to the environment than any other single source. Chloroform is produced during the bleaching of wood pulp, a process that whitens the final paper product. Rehm et al. (1982) based their estimation on information contained in a number of documents concerned with the pulp and paper industry (U.S. EPA, 1980f; NCASI, 1977; Metcalf and Eddy, Inc., 1972; TAPPI, 1963). From the operating conditions, analytical data, and production steps detailed in these documents, Rehm et al. (1982) determined the quantity of chloroform produced for each of nine different types of mills for which monitoring data existed and applied these values to all mills for which no values from sampling existed. The authors determined that chloroform is emitted at three different stages: into the air during the bleaching process itself; into the air during the detention time in wastewater treatment plants; and into the water from treatment plant effluent. The amount of chloroform produced annually was calculated to be 128 metric tons (282 x 10^3 lb) released to air during bleaching operations, 3985 metric tons (8.78 x 10^6 lb) released to air from wastewater detention (4113 metric tons to air), and 298 metric tons (657 x 10^3 lb) discharged into water from treatment plants (4411 metric tons or 9.72 x 10^6 lb total).

3.4.1.2.6. Chloroform production from combustion of leaded gasoline. Chloroform has been reported to be a component of automobile exhaust (Harsch et al., 1977). Its presence is reportedly the result of using ethylene dichloride and ethylene dibromide as lead scavengers in leaded gasoline (Lowenbach and Schlesinger Associates, 1979). Rehm et al. (1982) cite other sources which state that chloroform is not formed during the combustion of leaded fuel containing ethylene dichloride. The Emissions Testing and Characterization Branch of EPA's Environmental Sciences Research Laboratory measured chlorocarbon emissions from automobiles using leaded gasoline and found no chloroform. Rehm et al. (1982) then reason that even if chloroform were present in automobile exhaust, the decrease in the usage of leaded gasoline will decrease the amount of chloroform produced.

The authors cite an EPA report (U.S. EPA, 1982a) which states that leaded gasoline consumption is expected to drop by >75%, from 34 x 10^9 gallons to 8.3 x 10^9 gallons/year. Rehm et al. (1982) used the estimate of Wagner et al. (1980) that 1% of the ethylene dichloride in gasoline would be converted to chloroform, and the new lead phase-down regulations (U.S. EPA, 1982b) to calculate an annual emission rate of 180 metric tons (397 x 10^3 lb) in 1983 and 44 metric tons (97 x 10^3 lb) by 1990.

3.4.1.2.7. Chloroform formation during atmospheric trichloroethylene decomposition. Trichloroethylene is a major industrial solvent used principally for vapor degreasing of fabricated metal parts (66%) (Chemical Marketing Reporter, 1981), and the majority of each year's production is used for replacement of evaporative loss to the environment (Rem et al., 1982). The postulated formation of chloroform during the atmospheric decomposition of trichloroethylene is based on laboratory experiments in which trichloroethylene, NO_2, H_2O, and a hydrocarbon mixture were irradiated with light having the intensity and spectral distribution of the lower troposphere (U.S. EPA, 1976). Dichloroacetylchloride, phosgene, chloroform, and HCl were detected as products. Rehm et al. (1982) did not describe the method they used to determine the amount of chloroform produced from this reaction; however, they estimated 780 metric tons of chloroform are produced annually.

3.4.1.2.8. Miscellaneous indirect source. Wagner et al. (1980) have listed a number of other indirect sources of chloroform that defy quantification. These sources are: chlorination wastewaters from the textile industry, the food processing industry, breweries, combustion of tobacco products treated with chlorination pesticides, thermal decomposition of plastics, biological production in red marine algae, and the reaction of chlorinated pollutants with humic substances in natural waters.

3.4.1.2.9. Summary of indirect production. Chloroform is produced and emitted into the environment from a variety of indirect sources. These sources account for ≈84% of all chloroform air emissions, 98% of water discharges, and 36% of all land discharges (84.6% of all environmental releases). The environmental discharges of chloroform from indirect sources are summarized in Table 3-5.

3.4.2. Emissions from Use

Chloroform is used consumptively for the production of chlorodifluoromethane or Fluorocarbon-22 (accounts for 90% of domestic 1982 production, 60% for refrigerants, 30% for fluoropolymer) and for exports (3% in 1982) (Chemical Marketing Reporter, 1983). It is used nonconsumptively as an extraction solvent; as a solvent for penicillin, alkaloids, vitamins, flavors, lacquers, floor polishes, artificial silk manufacture, resins, fats, greases, gums, waxes, adhesives, oils, and rubber; as a dry cleaning agent; as an intermediate in pesticide and dye manufacture; and as a fumigant ingredient (Rem et al., 1982; DeShon, 1979; Merck Index, 1976). The great majority of chloroform used nonconsumptively is emitted into the environment since (except for expansions) the chloroform purchased for these uses is make-up solvent used to replace that amount not recovered from processes (Wagner et al., 1980).

TABLE 3-5. CHLOROFORM DISCHARGES FROM INDIRECT SOURCES

	Environmental release (metric tons/year)			
Source	Air	Water	Land	Total
Pulp and paper mills	4113	298	0	4411
Drinking water chlorination	0	1900	0	1900
Ethylene dichloride manufacture	760	---[a]	217	977
Trichloroethylene photodegradation	780	0	0	780
Municipal wastewater chlorination	0	320	0	320
Cooling water chlorination	190	72	0	262
Automobile exhaust	180	0	0	180
TOTAL	6023	2590	217	8830

[a] Minor releases possible.

SOURCE: Rehm et al., 1982.

3.4.2.1. Emissions from Pharmaceutical Manufacturing--Chloroform is used as an extraction solvent during the manufacture of some antibiotics and steroids, and during the manufacture of certain other biological and natural pharmaceuticals (Rem et al., 1982). It is also used as a chemical intermediate. Based on a Pharmaceutical Manufacturing Association (PMA) Survey, Rehm et al. (1982) reported that ≃1000 metric tons of chloroform are released into the environment (no year was specified; the PMA report was dated 1978). The distribution was as follows: 57.0% (570 metric tons) to air, 4.6% (46 metric tons) to water, and 38.4% (384 metric tons) to land.

3.4.2.2. Emissions from Fluorocarbon-22 Production--The single largest use of chloroform is for Fluorocarbon-22 production (Chlorodifluoromethane, $CHClF_2$). Fluorocarbon-22 producers and production sites are listed in Table 3-6. Chloroform release to the environment can occur from process emissions, fugitive emissions, and storage emissions (Rem et al., 1982). Rehm et al. (1982) reported that the first source listed does not represent a significant source of chloroform emissions based on the design of Fluorocarbon-22 production facilities and the process description.

Storage emission estimates were based on U.S. EPA (1980g) for chloroform feedstock storage in fixed roof tanks. Rehm et al. (1982) reported that the Allied Chemical facility at El Segundo, California, uses a control system that results in complete capture of chloroform vapors. Du Pont (and all others by assumption) use refrigerated condensers that reduce the uncontrolled emission factor of 2.5 kg/metric ton by 66%. Fugitive emissions from leaks in valves, pumps, compressors, and relief valves were estimated to result in an uncontrolled emission rate of 0.75 kg/metric ton. Total emissions to air were calculated by Rehm et al. (1982) to be 139 metric tons/year by multiplying the emission factors by 1980 Fluorocarbon-22 production (97,500 metric tons).

No estimate was made for emissions to wastewater because of a lack of data. Emissions to land were based on the reported practice of landfilling spent catalyst by Allied. Rehm et al. (1982) assumed a catalyst contamination level of 10% and a total emission of 2.0 metric tons/year.

3.4.2.3. Emissions from Hypalon® Manufacture--Hypalon® is a chemically resistant synthetic rubber made by substituting chloride and sulfonyl groups onto polyethylene. The process involves dissolving polyethylene in chloroform followed by reaction with chlorine and sulfur dioxide. Based on a

TABLE 3-6. CHLORODIFLUOROMETHANE PRODUCERS AND PRODUCTION SITES

Producer	Production site
Allied Corp. Allied Chem. Co.	Baton Rouge, LA Danville, IL El Segundo, CA
E.I. du Pont de Nemours and Co., Inc. Petrochems. Dept. Freon® Products Div.	Deepwater, NJ Louisville, KY Montague, MI
Pennwalt Corp. Chems. Group Fluorochemicals Div.	Calvert City, KY

SOURCE: SRI International, 1983.

Du Pont report, Rehm et al. (1982) estimated that 54.9 metric tons of chloroform were emitted into the air from Hypalon® manufacture in 1980, based on an emission inventory conducted by the Texas Air Control Board, Austin, Texas. No information was available for water or land emissions.

3.4.2.4. Chloroform Emissions from Grain Fumigation--Chloroform is a registered pesticide for use on certain insects that commonly infest stored raw bulk grains and is present in only one product (Rem et al., 1982). This product, Chlorofume® FC.30 Grain Fumigant (Reg. No. 5382-15), marketed by Vulcan Materials Company, contains 72.2% chloroform, 20.4% carbon disulfide, and 7.4% ethylene dibromide. Originally registered in 1968, the EPA issued a "Notice of Presumption Against Continued Registration of a Pesticide Product -- Chloroform (Trichloromethane)" in 1976 because of oncogenic effects in rats and mice (U.S. EPA 1982c). Continued study resulted in returning it to the normal registration process (U.S. EPA, 1982c). Based on a personal communication with D. Lindsay of Vulcan Materials, Rehm et al. (1982) estimated that between 10,000 and 12,000 gallons of chloroform per year were used in grain fumigants in the United States. Vulcan reported 1981 sales of 7000 gallons of Chlorofume® in 1981 or 5054 gallons (19,131 L). Based on its density, 28,400 kg (28.4 metric tons) was released to the environment (air) in this way.

3.4.2.5. Chloroform Losses from Loading and Transportation--Rem et al. (1982) estimated chloroform losses from loading ships, barges, tank cars, and tank trucks. The method was based on the degree of chloroform saturation of the air expelled from tanks during filling, temperature, vapor pressure, control efficiency, and filling methods as described by U.S. EPA (1979b) and Environment Reporter (1982). The U.S. mode of transportation was taken from Sax (1981) as follows: rail, 40.3%; barge, 47.8%; and truck, 11.9%. Loading losses were calculated to be 40.9 metric tons (90,200 lb).

Transit losses result from temperature and barometric pressure changes. The losses were assumed to be the same for barges, tank trucks, and rail cars and were estimated from the following equation:

$$L_T = 0.1\ PW$$

where LT = transit loss, lb/week - 10^3 gal transported

P = true vapor pressure of transported liquid, psia

W = density of condensed vapors lb/gal

No reference or justification for the use of the formula was presented (and commenters have noted the calculation for losses may be high because the method assumes a constant concentration throughout the vapor space). By this method, and assuming 1 week transit time, Rehm et al. (1982) calculated that

49.2 metric tons (0.11 x 10^6 lb) chloroform were lost to the air using 1980 production values.

3.4.2.6. Miscellaneous Use Emissions--Rem et al. (1982) cited the previous materials balance (Wagner et al., 1980) in predicting the emissions from chloroform contamination of methyl chloride, methylene chloride, and carbon tetrachloride. Chloroform is present to some extent in these products since they are all made by the same process. Assuming a contamination level of 7.5 ppm, 17.5 ppm and 150 ppm for methyl chloride, methylene chloride, and carbon tetrachloride, respectively, Rehm et al. (1982) estimate that releases to air, land and water would be 9.8, 0.6, and 0.15 metric tons, respectively.

Chloroform is also used in a variety of products (see Section 3.4.2) and as a general solvent. Rehm et al. (1982) estimate that, while these uses are generally declining, laboratory uses in particular may account for 8.5% of production or 14,200 metric tons of chloroform. Rehm et al. (1982), however, estimated the range of uncertainty to be ± 50%, and industry commenters have stated that laboratory use of chloroform is very minimal.

3.4.2.7. Summary of Chloroform Discharges from Use--Chloroform discharges from manufacturing facilities that use chloroform as a process ingredient account for 12% of all chloroform emissions to air, 1.8% of water discharges, and 63% of all land discharges, or 12.7% of chloroform discharges to all media. Table 3-7 summarizes chloroform discharges to all media.

3.4.3. Summary

Chloroform is produced by direct and indirect processes. Direct production accounts for 184 million kg annually, while indirect production accounts for ≈8.8 million kg annually.

Direct production of chloroform and processes associated with its use (i.e., Fluorocarbon-22 production, Hypalon® manufacture, loading and transit

TABLE 3-7. CHLOROFORM DISCHARGES FROM USE

Source	Environmental Release (metric tons/year)			
	Air	Water	Land	Total
Pharmaceuticals	570	46	384	1000
Chlorodifluoromethane manufacture	139	--[b]	2	141
Loading and transit losses	90.1	0	0	90.1
Hypalon® manufacture	54.9	--[a]	--[a]	54.9
Grain fumigation	28.4	0	0	28.4
Secondary product contamination	9.8	0.6	0.2	10.6
Total	892.2	46.6	386.2	1325.0

[a] Minor releases possible

SOURCE: Rehm et al., 1982.

losses, grain fumigation, pharmaceutical use) emit some 1.6 million kg to the environment. Virtually all of the indirectly produced chloroform may be emitted into the environment; assuming this to be so, the total amount of chloroform emitted is ≃10.4 million kg. This represents ≃5.6% of direct production. The relative source contributions from all quantifiable sources are listed in Table 3-8.

3.5. AMBIENT AIR CONCENTRATIONS

Monitoring data for a number of U.S. and world locations are presented in Table 3-9. For the most part, ambient concentrations remain <1000 ppt (1 ppt = 10^{-12}, v/v), and some <10 ppt. There are notable exceptions, however, although the reasons for this are not readily apparent.

TABLE 3-8. RELATIVE SOURCE CONTRIBUTION FOR CHLOROFORM

Source	Environmental Release (metric tons/year)							
	Air	% of Total[a]	Water	% of Total[a]	Land	% of Total[a]	Total	% of Total[a]
Pulp and paper mills	4113	39	298	2.9	0	0	4411	42
Drinking water chlorination	0	0	1900	18	0	0	1900	18
Pharmaceuticals	570	5.5	46	0.4	384	3.7	1000	10
Ethylene dichloride manufacuture	760	7.3	---[b]	----	217	2.1	977	9.4
Trichloroethylene photodegradation	780	7.5	0	0	0	0	780	7.5
Municipal wastewater chlorination	0	0	320	3.1	0	0	320	3.1
Cooling water chlorination	190	1.8	72	0.7	0	0	262	2.5
Methyl chloride chlorination	196	1.9	8	0.1	5.4	0.1	209.4	2.0
Automobile exhaust	180	1.7	0	0	0	0	180	1.7
Chlorodifluoromethane manufacture	139	1.3	----	----	2	0.02	141	1.4
Loading and transit losses	90.1	0.9	0	0	0	0	90.1	0.9
Methane chlorination	70.2	0.7	3.3	0.03	----	----	73.5	0.7
Hypalon® manufacture	54.9	0.5	----	----	----	----	54.9	0.5
Grain fumigation	28.4	0.3	0	0	0	0	28.4	0.3
Secondary product contamination	9.8	0.1	0.6	0.006	0.2	0.002	10.6	0.1
Laboratory usage[c]	----	----	----	----	----	----	----	----
TOTAL	7181.4	68.8	2647.9	25.4	608.6	5.8	10,438[a]	

[a] Values are rounded.
[b] Dashed lines indicate minor releases possible.
[c] Not included because of uncertainty.

SOURCE: Rehm et al. (1982).

TABLE 3-9. AMBIENT LEVELS OF CHLOROFORM

Location	Type of Site	Date	Analytical Method	Concentration (ppt, v/v) Max.	Min.	Average	Reference
Alabama							
Tuscaloosa	urban	2/77	GC-ECD	3000	100	800	Holzer et al., 1977
Talladega Forest	rural	2/77	GC-ECD	200	NR	100	Holzer et al., 1977
Arizona							
Phoenix	urban	4-5/79	GC-coulometry	514.0	27.1	111.4	Singh et al., 1981
California							
Stanford Hills	clean[a]	11/75	GC-ECD	217	12	33	Singh et al., 1979
Point Reyes	clean marine	12/75	GC-ECD	114	15	37	Singh et al., 1979
Los Angeles	urban	4-5/76	GC-ECD	724	23	102	Singh et al., 1979
Palm Springs	urban-suburban	5/76	GC-ECD	616	20	99	Singh et al., 1979
Yosemite	remote-high altitude	5/76	GC-ECD	24	12	17	Singh et al., 1979
Mill Valley	clean marine[a]	1/77	GC-ECD	36	4	25	Singh et al., 1979
Riverside	urban-suburban	4-5/77	GC-ECD	310	24	25	Singh et al., 1979
Badger Pass	remote-high altitude	5/77	GC-ECD	38	2	16	Singh et al., 1979
Point Arena	clean marine	5/77	GC-ECD	42	8	20	Singh et al., 1979
Point Arena	clean marine	8-9/78	GC-ECD	48	12	18	Singh et al., 1979
Los Angeles	urban	4/79	GC-ECD	223.5	24.3	88.2	Singh et al., 1981
Oakland	urban	6-7/79	GC-ECD	60.1	13.1	32.1	Singh et al., 1981
Delaware							
Delaware City	NS	7/74	GC-ECD	<10	<10	NR	Lillian et al., 1975a
Kansas							
Jetmore	remote-continental	6/78	GC-ECD	34	9	16	Singh et al., 1979

(continued on next page)

TABLE 3-9. (continued)

Location	Type of Site	Date	Analytical Method	Concentration (ppt, v/v)			Reference
				Max.	Min.	Average	
Maryland							
Baltimore	urban	7/74	GC-ECD	<10	<10	NR	Lillian et al., 1975a
Montana							
Western Montana	remote	3/76	GC-MS	NR	NR	9	Cronn and Harsch, 1979
Nebraska							
Reese River	remote-high altitude	5/77	GC-ECD	19	6	13	Singh et al., 1979
New Jersey							
Rutherford	urban	1978	GC-MS	31,000	NR	4600	Bozzelli and Kebbekus, 1979
Newark	urban	1978	GC-MS	7500	NR	3900	Bozzelli and Kebbekus, 1979
Piscataway	urban	1978	GC-MS	2900	NR	2200	Bozzelli and Kebbekus, 1979
Somerset (county)	urban	1978	GC-MS	11,000	NR	5000	Bozzelli and Kebbekus, 1979
Bridgewater township	rural	1978	GC-MS	NR	NR	ND[b]	Bozzelli and Kebbekus, 1979
Bound Brook	urban	3/76	GC-MS	NR	NR	854	Pellizzari, 1977
Patterson	urban	3/76	GC-MS	NR	NR	768	Pellizzari, 1977
Clifton	urban	3/76	GC-MS	NR	NR	1700	Pellizzari, 1977
Fords	urban	3/76	GC-MS	NR	NR	3422	Pellizzari, 1977
Newark	urban	3/76	GC-MS	NR	NR	7582	Pellizzari, 1977
Passaic	urban	3/76	GC-MS	NR	NR	854	Pellizzari, 1977
Hoboken	urban	3/76	GC-MS	NR	NR	427	Pellizzari, 1977
Seagrit	urban	6/74	GC-ECD	60	<10	40	Lillian et al., 1975a
Seagrit	urban	6/75	GC-ECD	50	5	35	Lillian et al., 1975b
Sandy Hook	urban	7/74	GC-ECD	63	<10	30	Lillian et al., 1975a
Sandy Hook	urban	7/75	GC-ECD	55	10	25	Lillian et al., 1975b

(continued on next page)

TABLE 3-9. (continued)

Location	Type of Site	Date	Analytical Method	Concentration (ppt, v/v) Max.	Min.	Average	Reference
Bayonne	urban	7/75	GC-ECD	15,000	<10	1030	Lillian et al., 1975a
New York							
Staten Island	urban	3/76	GC-MS	NR	NR	4268	Pellizzari, 1977
New York City	urban	6/74	GC-ECD	480	<10	160	Lillian et al., 1975a
New York City	urban	6/75	GC-ECD	450	10	200	Lillian et al., 1975b
White Face Mountain	remote	9/74	GC-ECD	250	<10	9	Lillian et al., 1975a
White Face Mountain	remote	9/75	GC-ECD	350	6	8	Lillian et al., 1975b
Niagara Falls	urban	NS	GC-MS	21,611	215	NR	Pellizzari et al., 1979
Ohio							
Wilmington	Air Force Base	7/74	GC-ECD	4800	<10	340	Lillian et al., 1975a
Wilmington	Air Force Base	7/75	GC-ECD	5000	20	480	Lillian et al., 1975a
Texas							
Houston	urban	6-7/77	GC-MS	11,034	Trace	NR	Pellizzari et al., 1979
Washington							
Pullman	rural	12/74 to 2/75	GC-MS	NR	NR	20	Grimsrud and Rasmussen, 1975
Pullman	rural	11/75	GC-ECD	NR	NR	43	Rasmussen et al.,1977
England							
Liverpool /Manchester	suburban	NS	GC-ECD	8^c	3^c	NR	Pearson and McConnell, 1975

(continued on next page)

TABLE 3-9. (continued)

Location	Type of Site	Date	Analytical Method	Concentration (ppt, v/v) Max.	Min.	Average	Reference
Organochlorine manufacturer	urban	NS	GC-ECD	40^c	$<10.1^c$	NR	Pearson and McConnell, 1975
Moel Faman, Flintshire	urban	NS	GC-ECD	0.4^c	$<0.1^c$	NR	Pearson and McConnell, 1975
Rannoch Moor, Argyllshire	urban	NS	GC	0.5^c	0.1^c	NR	Murray and Riley, 1973
Rural areas	rural	NS	GC	1.2	0.82	0.82	Murray and Riley, 1973
Ireland							
Cork	urban	1974	GC-ECD	NR	NR	26.5	Cox et al., 1976
Japan							
Kobe	NS	NS	GC-ECD	9400	300		Okuno et al., 1974
Atlantic Ocean							
Northeast Atlantic (Cape Blanc to Lands End) 34°19'N 13°32'W to 49°54'N 05°54'W		7-8/72	GC	0.96	0.14	0.35	Murray and Riley, 1973

[a] Subject Urban Transport.
[b] Detection Limits 10 ppt.
[c] ppb by mass.
NR = not reported; NS = not specified; ND = not detected.

Singh (1977) and Singh et al. (1979) have determined northern and southern hemisphere background concentrations as well as a global average. The hemispheric values they reported are 14 ppt for the northern hemisphere and <5 ppt for the southern hemisphere. This difference in hemispheric values suggests that the oceans are not a significant source of chloroform, but rather, that chloroform, for the most part, is anthropogenic. More recent research, however, suggests that just the opposite may be true. Khalil et al. (1983) have analyzed global concentrations of chloroform in the air and seawater, and concluded that the tropical oceans, at least, are a sizeable source of chloroform to the atmosphere. The reported atmospheric concentrations of chloroform ranged from 16 ppt at the South Pole to 45 ppt at Cape Meares, Oregon, with a ratio of concentrations between the northern and southern hemispheres of 1.6. Khalil et al. (1983) discuss the sources and sinks of chloroform in the atmosphere, construct a global mass balance equation, and conclude that chloroform in the atmosphere may be largely natural in origin, rather than mostly anthropogenic as previously thought.

An interesting point not presented in Table 3-9 is that chloroform concentrations above an inversion layer are significantly lower than concentrations below it. In Wilmington, OH, above an inversion layer, the chloroform concentration was <10 ppt, whereas below it the concentration was 120 ppt (Lillian et al., 1975a).

3.6. ATMOSPHERIC REACTIVITY

The principal atmospheric reactant responsible for the removal of chloroform is probably the hydroxyl radical (Atkinson et al., 1979; Graedel, 1978; Altshuller, 1980; Singh, 1977; Crutzen and Fishman, 1977). Hydroxyl radicals are formed in the lower atmosphere in at least two ways, first, by the photodissociation of ozone (λ <310) into O (^{1}D) atoms (Atkinson et al., 1979).These go on to react with either water, hydrogen or methane to form hydroxyl radicals. The second important source of hydroxyl radicals is the reaction of hydroperoxyl radicals with nitric oxide.

Hydroxyl radical reactions probably follow the course outlined below (Graedel, 1978):

$$CHCl_3 + HO \dashrightarrow \cdot CCl_3 + H_2O$$

$$\cdot CCl_3 + O_2 \dashrightarrow \cdot O_2CCl_3$$

$$\cdot O_2CCl_3 + NO \dashrightarrow \cdot OCCl_3 + NO_2$$

$$\cdot OCCl_3 \dashrightarrow COCl_2 \text{ (phosgene) } + Cl\cdot$$

$$COCl_2 + H_2O \dashrightarrow CO_2 + 2\ HCl$$

Pearson and McConnell (1975) found HCl and CO_2 as the only products of chloroform irradiation with UV (λ >290 nm) light. The half-life reported by these workers (23 weeks) was of the same order of magnitude as that calculated from the hydroxyl radical rate constant (11.5 weeks) (Singh et al., 1981).

Chloroform will not react photolytically in the troposphere; the UV cutoff for chloroform is 175 nm (i.e., it will not absorb light >175 nm). Callahan et al. (1979) calculated that roughly 1% of the tropospheric chloroform would diffuse eventually into the stratosphere, based on a lifetime of 0.2-0.3 years and a troposphere-to-stratosphere turnover time of 30 years.

3.7. ECOLOGICAL EFFECTS/ENVIRONMENTAL PERSISTENCE

3.7.1. Ecological Effects

3.7.1.1. Terrestrial--Data on the terrestrial ecological effects of chloroform are not available. Significant effects are not expected because chloroform is quite volatile and does not accumulate in terrestrial (or aquatic) environments, and is diluted rapidly and degraded to low concentrations in the troposphere (NAS, 1978). Conceivably, acute effects on wildlife can occur in the vicinity of major chloroform spills, but significant chronic effects from long-term exposure to low ambient levels is unlikely.

3.7.1.2. Aquatic--The toxicity of chloroform to aquatic organisms has been reviewed by the U.S. EPA (1980h). As summarized in Table 3-10, two freshwater fish (rainbow trout, bluegill) and one invertebrate (*Daphnia magna*) species have been acutely tested under standard conditions; LC_{50} concentrations ranged from 28,900 to 115,000 µg/L (Bentley et al., 1975; U.S. EPA, 1978a), and the trout was more sensitive than the bluegill. With stickleback, goldfish, and orange-spotted sunfish, anesthetization or death occurred after exposure to 97,000 to 207,000 µg/L chloroform for 30 to 90 minutes (Clayberg, 1917; Jones, 1947a; Cherkin and Catchpool, 1964). Only one test has been conducted with chloroform and saltwater organisms; the 96-hour LC_{50} for the pink shrimp was 81,500 µg/L (Bentley et al., 1975).

Embryo-larval tests with rainbow trout at 2 levels of hardness provided 27-day LC_{50} values of 2030 and 1240 µg/L (Birge et al., 1979). There was a 40% incidence of teratogenesis in the embryos at hatching (23 day exposure at 10,600 µg/L).

Bluegills bioconcentrated radiolabeled chloroform by a factor of 6 after a 14-day exposure, and the tissue half-life was <1 day (U.S. EPA, 1978a). This degree of bioconcentration and short biological half-life suggest that chloroform residues would not be an environmental hazard to consumers of aquatic life (U.S. EPA, 1980h).

TABLE 3-10. ACUTE AND CHRONIC EFFECTS OF CHLOROFORM ON AQUATIC ORGANISMS

Species	Duration	Concentration (μg/L)	Method	Effect	Reference
Cladoceran *Daphnia magna*	48-hr	28,900	S,U[a]	LC_{50}	U.S. EPA, 1978a
Rainbow trout *Salmo gairdneri*	96-hr	66,800	S,U	LC_{50}	Bentley et al., 1975
Rainbow trout *Salmo gairdneri*	96-hr	43,800	S,U	LC_{50}	Bentley et al., 1975
Bluegill *Lepomis macrochirus*	96-hr	115,000	S,U	LC_{50}	Bentley et al., 1975
Bluegill *Lepomis macrochirus*	96-hr	100,000	S,U	LC_{50}	Bentley et al., 1975
Pink shrimp *Penaeus duorarum*[b]	96-hr	81,500	S,U	LC_{50}	Bentley et al., 1975
Orangespotted sunfish *Lepomis humilis*	1-hr	106,890 to 152,700	NS	death	Clayberg, 1917
Goldfish *Carassius auratus*	30-60 min	97,000 to 167,000	NS	50% anesthetized	Cherkin and Catchpool, 1964
Threespine stickleback *Gasterosteus aculeatus*	90-min	207,648[c]	NS	anesthesia with recovery	Jones, 1947a
Ninespine stickleback *Pungitius pungitius*	NS	148,320 to 296,640	NS	Avoidance	Jones, 1947b

TABLE 3-10 (continued)

Species	Duration	Concentration (μg/L)	Method	Effect	Reference
Rainbow trout (embryo-larval) *Salmo gairdneri*	27 days[d]	2,030	F,M[e]	LC_{50} at 50 mg/L hardness	Birge et al., 1979
Rainbow trout (embryo-larval) *Salmo gairdneri*	27 days[d]	1,240	F,M[e]	LC_{50} at 200 mg/L hardness	Birge et al., 1979
Rainbow trout (embryo) *Salmo gairdneri*	23 days	10,600	F,M[e]	40% teratogenesis	Birge et al., 1979

[a] Static test, unmeasured concentration.
[b] Saltwater species.
[c] Corrected from vol/vol to μg/L.
[d] Exposures began within 20 minutes of fertilization and ended 8 days after hatching.
[e] Flow-through test, measured concentration.
hr = hour; min = minutes; NS = Not stated.

SOURCE: U.S. EPA, 1980h

3.7.2. Environmental Persistence

A number of researchers have reported the dominance of hydroxyl radical oxidations in the fate of chloroform in the atmosphere (see Section 3.6). Singh et al. (1981) calculated an atmospheric residence time for chloroform based on the NASA reviewed rate constant reported by Hampson (1980). They reported a 116-day (16.6-week) residence time for a hydroxyl radical concentration of 10^6 molecules/cm^3. This compares well to the observed 33-week lifetime of chloroform in a sunlit flask (Pearson and McConnell, 1975). This lifetime was based on experiments conducted in northwest England, which receives less intense sunlight than most of the U.S., and may account for its longevity.

According to recent hydroxyl radical measurements, tropospheric ambient air concentrations range from $\approx 10^6$ to 10^7 molecules/ml (Atkinson et al., 1979); models of the troposphere have suggested a concentration ranging between 2 to 6 x 10^5 molecules/m. (Crutzen and Fishman, 1977; Singh, 1977).

Table 3-11 summarizes the literature values for k_{OH}, the temperature of measurement, and the calculated lifetime based on the indicated hydroxyl radical concentration. If a hydroxyl radical concentration of 2 x 10^6 molecules/cm^3 (typical for summer months; winter concentrations are lower) (Singh et al., 1981) is assumed, most of the lifetimes calculated from the rate constants range between 0.2 to 0.5 years (69 to 181 days, 122 average).

Mabey and Mill (1978) critically reviewed hydrolysis data available in the literature. They determined that chloroform had a hydrolysis half-life of >3,000 years at pH 7 and 298 K. This is based on a base hydrolysis rate of 0.602 x 10^{-4} and a OH^- concentration of 10^{-7} in neutral water.

Dilling et al. (1975) and Dilling (1977) determined the volatilization half-life of chloroform from water. For a 1 ppm chloroform solution stirred

TABLE 3-11. VALUES FOR k_{OH}

kOH x 10^{14} cm^3 molecule-1 sec-1	K	[OH] x 10-5	Lifetime (years)	Reference
6.51	265	4	1.2	Singh et al., 1979
10.1	296			Howard and Evenson, 1976
16.8	298	10	0.19	Cox et al., 1976
11.4	298			Davis et al., 1976[a]
6.4	265	9	0.56	
7.4	273			Hampson, 1980[b]
10	298			

[a] $(4.69 \pm 0.71) \times 10^{-12} \exp - (2254 \pm 214/RT)$.

[b] Evaluated by NASA.

at 200 rpm, the time for 50% removal was 21.5 minutes (average); 90% removal was accomplished in 71 minutes. The addition of dry granular bentonite clay, dolomitic limestone, or peat moss had little effect on the evaporation rate. The rapid volatilization of chloroform was seen also by Jensen and Rosenberg (1975), who reported that 0.1-1.0 ppm solutions of chloroform in partly open sunlit aquaria lost 50-60% of the chloroform in 8 days as opposed to only 5% in closed aquaria. Pearson and McConnell (1975) suggested that the presence of chloroform in ambient waters may be from aerial transport and washout.

The potential for biodegradation of chloroform in the aquatic environment was examined by Pearson and McConnell (1975), who observed no aerobic biodegradation. Tabak et al. (1981) report "significant degradation with gradual adaptation" of chloroform in a static flask test. Under anaerobic conditions similar to those which may be present in lake sediments or groundwater, the reduction of chloroform without bacteria has been reported by Klecka and Gonsior (1984). In the presence of methanogenic bacteria, Bouwer and McCarty (1983) and Bouwer et al. (1981) report the degradation of chloroform with up to a 97% yield of CO_2. Similar observations by Wood et al. (1981) confirm these results.

An EXAMS model of the fate of chloroform in a pond, a river, and an oligotrophic lake and eutrophic lake revealed the dominant process in all cases to be volatilization. Input parameters included hydrolysis (2.5×10^{-9} hr^{-1}), octanol/water partition coefficient (91), vapor pressure (150.5 torr at 20°C), solubility (8200 ppm), Henry's Law Constant (2.88×10^{-3}), reaeration rate ratio (0.583), alkoxy radical rate constant (0.7 M^{-1} hr^{-1}) ($RO\cdot = 10^{14}$ M), and a stream loading of 1 g/hr. No photochemical or bacterial degradation parameters were entered since chloroform has no UV absorbance >175 nm, and virtually no bacterial degradation occurs with chloroform (Pearson and McConnell, 1975; Bouwer et al., 1981). Table 3-12 summarizes the EXAMS model generated fate of chloroform. Note that the EXAMS model does not include biodegradation rates.

3.8. EXISTING CRITERIA, STANDARDS, AND GUIDELINES

3.8.1. Air

The Occupational Safety and Health Administration (OSHA) currently limits occupational exposure to chloroform to a ceiling level of 50 ppm (29 CFR 1982a). This ceiling level is not to be exceeded in the workplace at any time. To protect against mild central nervous system depression, irritant effects, and fetal abnormalities (which were considered to occur at lower exposure levels than those causing liver injury), the National Institute for Occupational Safety and Health (NIOSH) recommended in 1974 that exposure to chloroform be limited to 10 ppm as a Time-Weighted Average (TWA) exposure for up to a 10-hour workday, 40 hour workweek. A ceiling level of 50 ppm was proposed for any 10-minute period (NIOSH, 1974). NIOSH lowered the recommended criterion to 2 ppm TWA in 1976 (NIOSH, 1977) in response to a positive NCI carcinogenesis bioassay (NCI, 1976). NIOSH recommended that exposure to halogenated anesthetic agents, including chloroform, be limited to 2 ppm because this is the lowest detectable level using the recommended sampling and analysis techniques, and not because a safe level of airborne exposure could be defined.

On the basis of recent reports of carcinogenicity and embryotoxicity, the American Conference of Governmental Industrial Hygienists (ACGIH) currently classifies chloroform as an Industrial Substance Suspect of Carcinogenic Potential for Man (ACGIH, 1981). The ACGIH recommends a Threshold Limit Value (TLV) of 10 ppm and a 15-minute Short-Term Exposure Limit (STEL) TWA of 50 ppm for chloroform.

TABLE 3-12. SUMMARY OF EXAMS[a] MODELS OF THE FATE OF CHLOROFORM[b]

	River	Pond	Oligotrophic Lake	Eutrophic Lake
Maximum total concentrations in water column (mg/L)	9.92×10^{-7}	2.50×10^{-3}	1.33×10^{-4}	1.26×10^{-4}
Maximum concentration in sediments (dissolved in pore water, mg/L)	9.85×10^{-7}	1.36×10^{-3}	5.82×10^{-6}	4.57×10^{-6}
Maximum concentration in Bios				
Plankton (μg/g)	2.58×10^{-5}	6.49×10^{-2}	3.45×10^{-3}	3.27×10^{-3}
Benthos (μg/g)	2.56×10^{-5}	3.53×10^{-2}	1.51×10^{-4}	1.19×10^{-4}
Maximum total concentration in sediment (mg/kg, dry weight)	3.19×10^{-6}	6.50×10^{-3}	2.83×10^{-5}	9.35×10^{-6}
Total steady accumulation (kg)	9.15×10^{-4}	5.43×10^{-2}	0.33	0.3
% in water column	96.93	91.9	99.95	99.94
% in sediments	3.07	8.1	0.05	0.06
Disposition				
chemical transformation (%)	0.00	0.00	0.00	0.00
biotransformation (%)	0.00	0.00	0.00	0.00
volatilization (%)	1.74	93.35	94.98	95.57
Volatilization half-life	36 hours	40 hours	10 days	9 days
exported (%)	98.26	6.65	5.02	4.43
export half-life	0.65 hours	566 hours	192 days	196 days
Mass flux from volatilization (kg/hr)	1.74×10^{-5}	9.33×10^{-4}	2.28×10^{-3}	2.29×10^{-2}
Self-purification time	37 hours	31 days	65 days	56 days

[a] Burns et al. 1981.
[b] Based on a stream load of 1.00 g/hr.

Foreign industrial air standards for chloroform include Bulgaria, 10 ppm; Czechoslovakia, 10 ppm (50 ppm for brief exposures); Finland, 50 ppm; Hungary, 4 ppm (20 ppm for brief exposures); Japan, 50 ppm; Poland, 10 ppm; Rumania, 10 ppm; Yugoslavia, 50 ppm; West Germany, 10 ppm (Utidjian, 1976).

3.8.2. Water

As discussed in the Ambient Water Quality Criteria Document for chloroform (U.S. EPA, 1980h), the EPA has proposed an amendment that would add to the National Interim Primary Drinking Water Regulations a section on the control of organic halogenated chemical contaminants. The proposed limit for total trihalomethanes in drinking water, which includes chloroform as the major constituent, is 100 μg/L. Although some estimates of cancer risk were performed, this limit was set primarily on the basis of technological and economic feasibility, and initially will apply only to water supplies serving >75,000 consumers. The basis and purpose of this regulation are discussed in a report that was prepared by the Office of Drinking Water (U.S. EPA, 1978b).

The U.S. EPA (1980h) recently derived cancer-based ambient water criteria for chloroform. Since zero level concentrations of chloroform will never be attainable in chlorine-treated water, levels that may result in incremental increases of cancer risk over the lifetime were estimated at risks of 1×10^{-5}, 10^{-6}, and 10^{-7}. The corresponding recommended criteria, which were derived with the tumor incidence data from the NCI bioassay with female mice (NCI, 1976), are 1.90, 0.19, and 0.019 μg/L, respectively, if exposure is assumed to be from the consumption of drinking water and fish and shellfish products and at 157 μg/L, 15.7 μg/L, and 1.57 μg/L, respectively, if exposure is assumed to be from the consumption of aquatic organisms only.

3.8.3. Food

Chloroform has been approved by the Food and Drug Administration (FDA) as a component of articles intended for use in contact with food (i.e., an indirect food additive). The use of chloroform in the food industry is summarized as follows:

Component of adhesives	CFR 1982b
Adjuvant substance required in the production of polycarbonate resins	CFR 1982c

Chloroform also has been exempted from the requirement of tolerance when used as a solvent in pesticide formulations that are applied to growing crops (CFR 1982d), or when used as a fumigant after harvest for barley, corn, oats, popcorn, rice, rye, sorghum (milo), or wheat (CFR 1982e).

3.8.4. Drugs and Cosmetics

The positive NCI carcinogenicity bioassay of chloroform (NCI, 1976) has prompted the FDA to restrict the use of chloroform in drug (CFR 1982f) and cosmetic (CFR 1982g) products.

3.9. RELATIVE SOURCE CONTRIBUTIONS

The sum of all the environmental releases of chloroform from all sources listed in Section 3.4.3 amounts to a total of 10,438 metric tons. All sources are summarized in Table 3-8 with the percent of the total emissions. Total emissions from all sources constitute about 5.6% of production (184,000 metric tons). Table 3-8 does not include estimated emissions from laboratory use. Rehm et al. (1982) suggested that these are potentially large but gave no numerical estimate.

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4. DISPOSITION AND RELEVANT PHARMACOKINETICS

4.1. INTRODUCTION

Considering that chloroform was the major anesthetic agent in use during the hundred years from its introduction by Simpson in 1847 (Waters, 1951; Snow, 1858; Simpson, 1847) until after the Second World War, there is relatively little detailed information about its pharmacokinetics and metabolism in man. This undoubtedly is due to the fact that until recently, specific and sensitive analytical methods were unavailable for the measurement of $CHCl_3$ and its metabolites at the concentrations in which they were likely to be present _in vivo_. Although chloroform as an anesthetic agent has been replaced by drugs with less cardiac and hepatic toxicity, it is still widely used in large bulk as an industrial solvent, as a chemical intermediary, and as a grain fumigant. Chloroform is present in the water supplies of many United States cities in concentrations reaching 311 μg/L, and also has been indentified as a contaminant of the air (U.S. Occupational Safety and Health Administration (OSHA), 1978; National Institute for Occupational Safety and Health (NIOSH), 1977b; Dowty et al., 1975; Symons et al., 1975). Accordingly, ordinary exposure to chloroform occurs from the workplace, food, drinking water, and ambient air (NIOSH, 1977b; Dowty et al., 1975; McConnell et al., 1975). Such exposure can be chronic by both oral and pulmonary routes, but at levels far below anesthetic concentrations (5000 to 10,000 ppm; 24.85 to 49.70 g/m^3). Nonetheless, chloroform has been detected in the breath of healthy people living in non-industrial environments (Conkle et al., 1975) and in post-mortem human tissue samples (McConnell et al., 1975).

4.2. ABSORPTION

Chloroform is rapidly and extensively absorbed through the lungs and from the gastrointestinal tract. Inhalation is considered the primary route of entrance into man for occupational exposure and air pollution. Absorption after oral ingestion is of particular interest since chloroform is a contaminative component of drinking water and foodstuffs. Significant absorption of chloroform through intact skin occurs only with liquid contact and not with vapors.

4.2.1. Dermal Absorption

Absorption of chloroform through the skin from direct liquid contact (immersion of hands or arms) is a slow process. Early studies (Torkelson et al., 1976; Schwenkenbecher, 1904; Witte, 1874) showed that chloroform does penetrate the skin and can be absorbed into the body by this route. Tsurata (1975, 1977) has studied the percutaneous absorption of a series of chlorinated organic solvents applied to a standard area of shaved abdominal mouse skin for 15 minute periods. Absorption was quantitated by presence of the compound in the total mouse body plus expired air, as determined by GC. For all solvents, percutaneous absorption linearly increased with time over the short exposure period and was directly related to water solubility. For chloroform the absorption rate was 329 μmoles/min/cm^2 skin, third highest of 8 solvents measured. Tsurata extrapolated this absorption rate to a calculation of the amount absorbed into the human body as the result of 1 min immersion of both hands (800 cm^2 area). The estimated amount absorbed, 19.7 mg/min, was equated to an inhalation exposure concentration of 2429 ppm for 1 min. Tsurata concluded that skin absorption from liquid contact could be a significant route of entry into the body for chloroform. More recently Jakobson et al. (1983) carried out similar experiments with guinea pigs for

10 chlorinated organic solvents (which however did not include chloroform). Liquid contact (skin area, 3.1 cm^2) was maintained for up to 12 hours and solvent concentration in blood was monitored during, and for some solvents after exposure. For these solvents, the blood elimination curves following dermal exposure were nonlinear, corresponding to a kinetic model involving at least two body compartments. Furthermore percutaneous absorption of these solvents, as reflected by blood concentration profiles, showed three different patterns that were related to water solubility. For solvents which were relatively hydrophilic [300 to 900 mg/100 ml water] the blood concentration increased steadily during the entire dermal exposure, indicating that absorption occurs faster than elimination by metabolism or pulmonary excretion. Chloroform with a water solubility of about 750 mg/100 ml water might be expected to be in this group.

4.2.2. Oral

The kinetics of gastrointestinal absorption of chloroform after oral ingestion have not been specifically studied; however, transmucosal diffusive passage occurs readily, as expected from its neutral and lipophilic properties (Tables 4-1 and 4-2), and as demonstrated by its biological effects produced by peroral administration of a wide range of dosages and dosing schedules for toxicity studies in rats, mice, guinea pigs, and dogs (Fishbein, 1979; Hill et al., 1975; Brown et al., 1974; Kimura et al., 1971; Klaassen and Plaa, 1967; Miklashevskii et al., 1966; Plaa et al., 1958; Eschenbrenner and Miller, 1945), teratologic studies in rats and rabbits (Thompson et al., 1974), and metabolism studies in mice, rats, rabbits, monkeys, and man (Brown et al., 1974; Taylor et al., 1974; Fry et al., 1972; Rubinstein and Kanics, 1964; Paul and Rubinstein, 1963). The rapid appearance of clinical symptoms following accidental and intentional

TABLE 4-1. PHYSICAL PROPERTIES OF CHLOROFORM AND OTHER CHLOROMETHANES[a]

		Ostwald solubility, 37°C		
	Vapor pressure at 25°C, torr	Water/ air	Blood/ air	Olive oil/ air
Dichloromethane	400	7.6	9.7	152
Chloroform	250	4.0	10.3	401
Carbon tetrachloride	100	0.25	2.4	361

[a] Conversion factors:

20°C; 750 mmHg   1 ppm in air = 4.97 μg/L = 4.97 mg/m^3
37°C; 760 mmHg   1 ppm in air = 4.69 μg/L = 4.69 mg/m^3

SOURCE: Sato and Nakajima, 1979.

TABLE 4-2. PARTITION COEFFICIENTS FOR HUMAN TISSUE AT 37°C

Tissue	Coefficient	Relative to blood
Blood	8.0	
Brain		
Grey matter	16	2.0
White matter	24	3.0
Heart	8	1.0
Kidney	11	1.4
Liver	17	2.1
Lung	7	0.9
Muscle	12	1.5
Fat tissue	280	35.0

SOURCE: Steward et al., 1973.

ingestion of chloroform has also been reported in man (Storms, 1973; Schroeder, 1965; Piersol et al., 1933).

Brown et al. (1974) and Taylor et al. (1974) found that ^{14}C-chloroform in olive oil given perorally to mice, rats, and monkeys (60 mg/kg) was essentially completely absorbed by virtue of a 93 to 98% recovery of radioactivity in exhaled air, urine, and carcass (Table 4-9). Absorption was rapid, with peak blood levels at 1 hour in mice and monkeys. In man, Fry et al. (1972) observed that ^{13}C-chloroform (0.5 g) in olive oil swallowed in a gelatin capsule resulted in rapid appearance of the stable isotope in exhaled breath (Table 4-8), with peak blood levels at 1 hour.

Withey et al. (1982) have investigated the effect of dosing vehicle on the intestinal absorption of chloroform in fasting rats (400 g) following intragastric intubation of equivalent doses (75 mg/kg) in about 4 ml of water or corn oil. The postabsorptive peak blood concentration averaged 6.5 times higher for water than corn oil (39 vs 6 μg/ml), while the time to initial peak blood concentration was essentially the same (5.6 vs. 6.0 min). Although the absorption from water vehicle exhibited one blood concentration peak, the absorption from corn oil showed two peaks in blood concentration at 6 and 40 minutes. The ratio of the areas under the blood concentration curves for 5 hours after dosing (AUC, 5 hr) was 8.7; water, corn oil. These results suggest that the absorption of chloroform with both vehicles is rapid. However, the rate and extent of absorption may be diminished, and the pattern of absorption altered, by intragastric intubation of high volumes (for a rat) of corn oil vehicle. A slower partitioning of lipophilic compounds dissolved in corn oil with mucosal lipids can be expected in comparison with a water vehicle. Furthermore, in contrast to aqueous absorption into the portal system and thence to the liver, corn oil and other liquids are extensively transported via mucosal lymphatic system which slowly drains by way of the left lymphatic thoracic duct into the systemic circulation via the superior vena cava. While these considerations are unlikely to affect the pharmacokinetics of chloroform in man in any practical way, they are of importance in relation to the modes of dosing employed in long-term carcinogenicity tests of chloroform and other lipophilic compounds.

4.2.3. Pulmonary Absorption

Chloroform has a relatively high vapor pressure (250 torr at 25°C; Table 4-1) and a high blood/air partition coefficient (8 to 10.3 at 37°C; Table 4-2); hence its vapor in ambient air is a primary mode of exposure and the lungs a principal route of entry into the body. The total amount absorbed via the lungs (as for all vapors) is directly proportional to (1) the concentration of the inspired air, (2) the duration in time of exposure, (3) the blood/air Ostwald solubility coefficient, (4) the solubility in the various body tissues, and (5) physical activity, which increases pulmonary ventilation rate and cardiac output. Hence, the basic kinetic parameters of the pulmonary absorption of chloroform and its equilibration in the body are as valid for low concentrations expected in the ambient environment as for the high vapor concentrations associated with its use as an anesthetic (5000 to 10,000 ppm; 24.85 to 49.70 g/m^3) (Smith et al., 1973; Morris, 1951; Waters, 1951). These parameters have not been as well studied as they have for modern anesthetics like halothane (Fiserova-Bergerova and Holaday, 1979) or even for other common halogenated hydrocarbon solvents like trichloroethylene, methylene chloride, or methylchloroform.

The earliest attempt at controlled studies of pulmonary absorption of chloroform in man were conducted by Lehmann and Hasegawa (1910). These investigators calculated retention values for chloroform (% inspired air concentration of chloroform retained in the body) from differences between inspired and expired air concentrations (analyzed by alkali hydrolysis with chloride titration). As expected, initial retention values were high, and decreased with exposure duration as total body equilibrium with inspired air concentration was approached (Table 4-3). Both the rate of uptake to equilibration and the final retention value achieved are related to the solubility of chloroform in blood (blood/air partition coefficient). Figure 4-1 illustrates for chloroform and other anesthetic vapors that the greater the Ostwald solubility coefficient for a vapor agent, the less rapidly equilibrium occurs. From the data of Lehmann and Hasegawa (Table 4-3) and recent data of Smith et al. (1973) concerning blood levels of concern during anesthesia as shown in Figure 4-2, total body equilibrium with inspired chloroform concentration requires at least ≥2 hours in normal man at resting ventilation rate and cardiac output. The retention value at equilibrium suggested by the Lehmann and Hasegawa (1910) data is ≃65%, and is 67% as calculated from the data of Smith et al. (1973). The difference, 33 to 36%, represents body elimination of chloroform by routes other than pulmonary (primarily by metabolism). The percent retention value is independent of the inspired air concentration at equilibrium.

The magnitude of chloroform pulmonary uptake into the body (dose, body burden) is directly related to the concentration of chloroform in the inspired air and to the duration of exposure. The total amount retained in the body during inhalation exposure can be estimated by multiplying percent retention (R) by the volume of air inspired during the exposure period, or:

$$\text{Amount uptake} = (C_I - C_A) \cdot V \cdot T$$

where V is ventilation rate (L/minute), T is exposure period (minute), and C_I and C_A are inspired air concentration and end alveolar air concentration,

TABLE 4-3. RETENTION AND EXCRETION OF CHLOROFORM BY MAN DURING AND AFTER INHALATION EXPOSURE TO ANESTHETIC CONCENTRATIONS

	Subject (Inspired air conc., ppm)		
	1 (4448)	2 (4920)	3 (4407)
	Retention, %		
Exposure period, min			
0 to 5	74.5	68.4	80.0
5 to 10	72.4	61.6	74.2
10 to 15	68.6	51.2	76.9
15 to 20	67.6	50.2	74.6
20 to 25	NR	NR	74.2
25 to 30	NR	NR	73.8
	Excretion, mg/L expired air		
Postexposure, min			
0 to 10	NR	NR	1.70
10 to 20	NR	NR	0.97
20 to 30	NR	NR	0.85

NR = not reported; min = minutes.

SOURCE: Lehmann and Hasegawa, 1910.

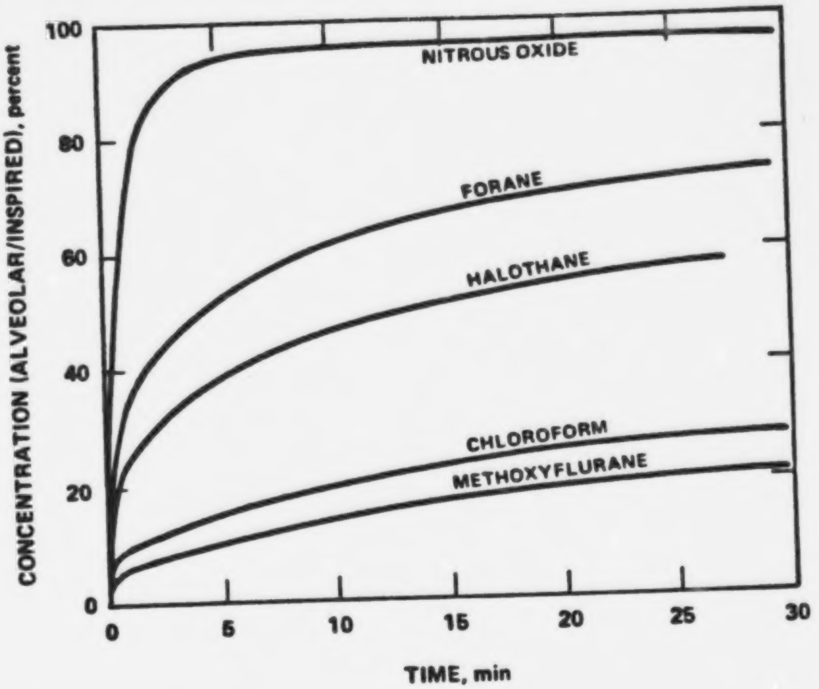

Figure 4-1. Rate of rise of alveolar (arterial) concentration toward inspired concentration for five anesthetic agents of differing Ostwald solubilities (blood/air partition coefficients): nitrous oxide, 0.47; forane, 1.4; halothane, 2.4; chloroform, 8; and methoxyflurane, 11. Note rate of alveolar chloroform rise is less than that of halothane with a smaller Ostwald coefficient and greater than that of methoxyflurane with a larger coefficient.

Source: Munson (1973)

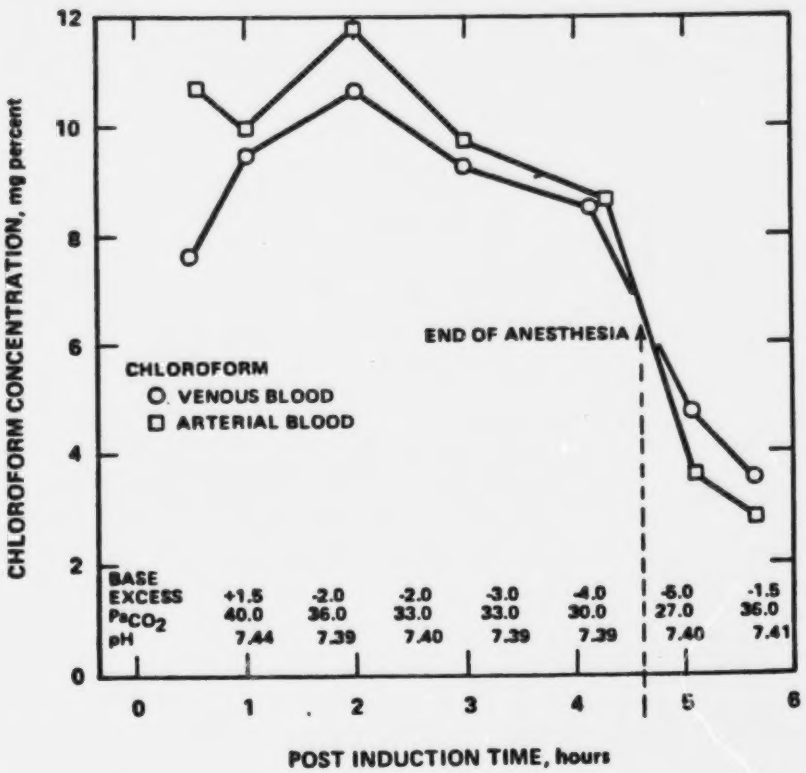

Figure 4-2. Arteriovenous blood concentrations of a patient during anesthesia with chloroform. Note anesthetic blood concentration for chloroform, the decreasing difference between arterial and venous concentrations at 2 to 3 hours, indicating whole-body equilibrium, and the rapid fall of blood concentration with termination of chloroform exposure.

Source: Smith et al. (1973).

respectively. Physical activity increases uptake by increasing the ventilation rate, V, and the cardiac output which influences rate of distribution to the various tissues of the body.

During inhalation of chloroform (and in the post exposure elimination phase), the arterial blood concentration of chloroform is directly proportional to inspired air concentration (and end alveolar air concentration). This fixed relationship is defined by the blood/air partition coefficient in comparison to other solvents (Sato and Nakajima, 1979) (Table 4-1), and hence, for equivalent ambient air exposure concentrations, the blood concentration of chloroform is proportionally higher. For inspired air concentration required for surgical anesthesia (8,000 to 10,000 ppm; 39/76 to 49.70 g/m^3), Smith et al. (1973) observed a mean arterial blood chloroform concentration for 10 patients of 9.8 mg/dl with a range of 7 to 16.5 mg/dl (Figure 4-2), and Morris (1951) found similar values for his patients. For inspired air concentrations less than anesthetic levels, for example low vapor concentrations of 10 to 100 ppm (49.7 to 497 mg/m^3), blood chloroform concentrations are lower in direct proportion.

The amount of pulmonary absorption of chloroform is also influenced by total body weight and by the total fat content of the body (average body fat content is 8% of body weight) (Geigy Scientific Tables, 1973). The capacity of adipose tissue to absorb chloroform in vivo is determined by the product of adipose tissue weight and lipid solubility of chloroform. The lipid solubility of chloroform is relatively high for this haloalkane (olive oil/air, 401; Tables 4-1 and 4-2), and also, the adipose tissue/blood partition coefficient is high (280 at 37°C); therefore, the uptake and storage of chloroform in adipose tissue can be substantial, and this uptake and storage is increased with excess body weight and obesity.

4.3. TISSUE DISTRIBUTION

Chloroform, after pulmonary or peroral absorption, is distributed into all body tissues. The compound crosses the placental barrier, as indicated by embryotoxicity and teratogenicity in mice, rats, and rabbits after oral and inhalation dosing (Murray et al., 1979; Dilley et al., 1977; Schwetz et al., 1974; Thompson et al., 1974). It has been found in fetal liver (von Oettingen, 1964). Chloroform can be expected to also appear in human colostrum and mature breast milk, since it has been found in fresh cow's milk and in high content in cheese and butter (Table 4-4).

As to be expected from the lipophilic nature of chloroform and modest water solubility (Table 4-1), highest concentrations are detected in tissues with higher lipid content; relative tissue concentrations are reflected by individual tissue/blood partition coefficients. Coefficients for human tissues, given in Table 4-2, indicate that relative tissue concentrations are expected in the order of adipose tissue > brain > liver > kidney > blood. The absolute amounts of chloroform detected in these tissues at any given time are proportional to the body dose (i.e., to the concentration in the inspired air and duration of inhalation or to the oral dose, partition coefficient, and to the tissue compartment size).

Gettler (1934) and Gettler and Blume (1931), using a modified Fujiwara analytical method, determined the chloroform content of the brain, lungs, and liver of nine patients who died during surgical anesthesia (presumably 5000 to 10,000 ppm; 24.85 to 49.50 g/m^3 inspired air) as: brain, 120 to 182; lung, 92 to 145; and liver, 65 to 88 mg/kg tissue wet weight. Even higher values (372 to 480 mg/kg in brain tissue) were detected in seven cases of death due

TABLE 4-4. CHLOROFORM CONTENT IN UNITED KINGDOM FOODSTUFFS AND IN HUMAN AUTOPSY TISSUE

Chloroform in U.K. foodstuffs	
Foodstuff	Chloroform, µg/kg
Dairy produce	
Fresh milk	5
Cheshire cheese	33
English butter	22
Hens' eggs	1.4
Meat	
English beef (steak)	4
English beef (fat)	3
Pig's liver	1
Oils and fats	
Margarine	3
Olive oil (Spanish)	10
Cod liver oil	6
Vegetable cooking oil	2
Beverages	
Canned fruit drink	2
Light ale	0.4
Canned orange juice	9
Instant coffee	2
Tea (packet)	18
Fruit and vegetables	
Potatoes (S. Wales)	18
Potatoes (N.W. England)	4
Apples	5
Pears	2
Tomatoes	2
Fresh bread	2

Chloroform in human autopsy tissue			
Age of subject	Sex	Tissue	Chloroform, µg/kg (wet tissue)
76	F	Body fat	19
		Kidney	2
		Liver	5
		Brain	4
76	F	Body fat	5
		Kidney	5
		Liver	1
		Brain	2
82	F	Body fat	67
		Liver	8.7
48	M	Body fat	67
		Liver	9.5
65	M	Body fat	64
		Liver	8.8
75	M	Body fat	65
		Liver	10.0
66	M	Body fat	68
74	F	Body fat	52

SOURCE: McConnell et al., 1975.

to excessive administration of chloroform (Gettler, 1934). The blood concentration during surgical anesthesia has been recently determined (by GC) to range from 70 to 165 mg/L in 10 patients (average, 98) by Smith et al. (1973). These tissue concentrations are in general agreement with the tissue/blood partition coefficients summarized from the literature by Steward et al. (1973) and given in Table 4-2.

In contrast to the high tissue levels of chloroform detected in response to inspired air concentrations required for anesthesia, McConnell et al. (1975) recently analyzed post-mortem tissue from eight persons, four males and four females, with an age range of 48 to 82, living in the United Kingdom in ordinary non-industrial circumstances, for chloroform and other halogenated compounds (carbon tetrachloride, trichloroethylene, perchloroethylene, hexachlorobutadiene). Significant tissue levels of three chlorinated hydrocarbons were detected. Chloroform levels, µg/kg wet tissue weight, were as follows: body fat, 5 to 68 (average of 51); liver, 1 to 10 (average of 7.2); kidney, 2 to 5; and brain, 2 to 4 (Table 4-4). Presumably, these tissue levels of chloroform were derived from air, foodstuff (Table 4-4), and drinking water contamination (OSHA, 1978; Dowty et al., 1975; Symons et al., 1975).

There have been few controlled exposure studies in animals investigating the distribution of chloroform in body tissues and determining dose-dependent tissue concentrations. Chenoweth et al. (1962) determined blood and tissue concentrations of chloroform in two normal fasted dogs after 2.5 hours of surgical anesthesia. Concentration of chloroform in the inhaled stream during anesthesia was not determined, but anesthesia was judged to be satisfactory at an arterial level of 45 to 50 mg/dl. Blood and tissue chloroform levels were determined by infrared spectroscopy after tissue extraction in cold carbon disulfide and distillation. Table 4-5 shows the relative concentration of chloroform in body tissues. The highest concentrations were detected in fat tissue, some 10-fold greater than blood, and in adrenals (4-fold greater than blood); the concentrations in brain, liver, and kidney were similar to blood.

Cohen and Hood (1969) used low-temperature whole-body autoradiography to study the distribution of ^{14}C-chloroform in mice. Individual mice were administered 2.4 μl of ^{14}C-labeled chloroform by inhalation over a 10-minute period. The animals were sacrificed 0, 15, or 120 minutes after exposure. Autoradiography of mice killed immediately after inhalation showed the highest concentration of radioactivity in body fat and liver, while lesser and relatively uniform amounts were seen in blood, brain, lung, kidney, and muscle. By 120 minutes after exposure, a considerable decrease in total radioactivity occurred, which was principally confined to liver, duodenum, and fat. A mottled appearance in the liver suggested a segmental or localized distribution. Biopsy specimens were taken from selected tissues in each animal and radioactivity determined by scintillation counting. Table 4-6 shows the distribution of radioactivity (chloroform and metabolites) in these tissues, and tissue/blood concentration ratios. Following sacrifice, after 10 minutes of exposure, most tissues approach a unit concentration with blood. However, in both fat and liver, the concentration exceeds unity. By 15 minutes, the ratio of radioactivity in brown fat reaches its peak at 15 times that detected in blood. The relative concentration of radioactivity in the liver continues to increase until the termination of the experiment at 120 minutes, when it reaches a final value 6.7 times in excess of that in the blood. Kidney and lung tissues also increased in relative concentration over the 2-hour period to a value of 1.53

TABLE 4-5. CONCENTRATION OF CHLOROFORM IN VARIOUS TISSUES OF TWO DOGS AFTER 2.5 HOURS OF ANESTHESIA

	Dog A	Dog B
	µg/g wet tissue weight ± 5%[a]	
Arterial blood	275	397
Brain	298	392
Adrenal (total)	1185	1305
Fat, omentum	2820	1450
Right ventricle	214	314
Skeletal muscle	189	155
Lung	147	336
Liver	282	290
Spleen	237	255
Kidney	225	226
Bile	209	205
Thyroid	460	760
Pancreas	296	350
Urine	57	73

[a] Chloroform concentration was determined by infrared spectrometry after tissue extraction.

SOURCE: Chenoweth et al., 1962.

TABLE 4-6. CONCENTRATIONS OF RADIOACTIVITY (CHLOROFORM PLUS METABOLITES) IN VARIOUS TISSUES OF THE MOUSE (NMRI)[a]

	Total radioactivity (counts/min/mg)			Tissue/blood ratio		
Tissue	0 min	15 min	120 min	0 min	15 min	120 min
Blood	260 ± 22.0	103 ± 17.5	37 ± 4.0	1.00	1.00	1.00
Brain	217 ± 16.4	112 ± 9.9	23 ± 2.7	0.84	1.12	0.63
Muscle	288 ± 44.4	110 ± 5.1	26 ± 6.9	0.87	1.07	0.70
Lung	262 ± 24.1	149 ± 12.6	53 ± 8.2	1.01	1.44	1.43
Kidney	284 ± 35.0	145 ± 21.6	56 ± 8.0	1.08	1.41	1.53
Liver	407 ± 36.9	208 ± 9.3	250 ± 17.9	1.56	2.10	6.76
Fat	1674 ± 201	953 ± 92.7	266 ± 30.1	6.42	9.25	7.18
Brown fat	3158 ± 384	1490 ± 98.4	211 ± 40.3	12.12	14.70	5.70

[a] Animal sacrifices were at 0, 15, or 120 minutes following 10-minutes inhalation of chloroform. Data represent duplicate determinations in each of two animals at each time sequence (± S.E).
Min = minutes.

SOURCE: Cohen and Hood, 1969.

and 1.43 of blood, respectively. The increasing ratios of liver and kidney/blood radioactivity represent a continued accumulation of metabolites within these organs. High body fat/blood concentrations shows that adipose tissue represents an important storage site, prolonging retention of chloroform in the body.

Whole-body autoradiography was also carried out by Brown et al. (1974) on male and female Sprague-Dawley rats and squirrel monkeys given ^{14}C-chloroform perorally (60 mg/kg). Male and female rats killed 3 hours after dosage showed no apparent sex difference in distribution of radioactivity. Radioactivity was greatest in body fat and liver, while lesser amounts were seen in blood, brain, lung, kidney, and muscle. Squirrel monkeys showed a similar distribution, with the exception that high concentrations of radioactivity were present in the bile and increased with time. Examination of bile extract by gas-liquid chromatography showed the bile radioactivity was unchanged chloroform, indicating an excretion of chloroform by the biliary route in the monkey.

Brown and his colleagues (Taylor et al., 1974) also investigated the tissue distribution of chloroform in 3 strains of mice (CF/LP, CBA, and C57) by whole-body autoradiography after oral dosing (60 mg/kg ^{14}C-chloroform). In the male mice of the three strains examined 3 hours after dosing, the greatest amounts of radioactivity appeared in liver and kidneys, and lesser amounts in renal cortex but not medulla. Female mice showed greatest radioactivity in liver, intestine, and bladder, with much less radioactivity in kidney and little differentiation between renal cortex and medulla. The same general patterns were observed 5, 7, and 24 hours after dosing. Biopsy samples of these tissues were taken, and radioactivity determined by scintillation counting. Table 4-7 shows the distribution of ^{14}C-chloroform

TABLE 4-7. TISSUE DISTRIBUTION OF ^{14}C-CHLOROFORM RADIOACTIVITY IN CF/LP MICE AFTER ORAL ADMINISTRATION (60 mg/kg)[a]

	Mean DPM/100 mg wet weight (SEM)	
Tissue	Male (6)	Female (6)
Liver	18,157 (1898)	21,535 (2097)
Kidney	13,759 (1047)	3,920 (533)
Brown fat	1,011 (80)	1,074 (54)
Blood	2,910 (423)	2,906 (457)

[a]Similar results were obtained for CBA and C57 strains.

SOURCE: Taylor et al., 1974.

radioactivity in male and female mice killed 5 hours after dosing. There is a 3.5-fold difference between the activity present in the male and female kidneys of each strain. Male mice had greater activity in the kidneys, but female mice showed relatively greater activity in the liver. This sex difference in distribution was abolished by castration or testosterone administration to female mice. The sex difference in tissue distribution of chloroform and its metabolites may relate to the nephrotoxic effect of chloroform that occurs in male mice but not in female mice (Bennet and Whigham, 1964; Culliford and Hewitt, 1957; Hewitt, 1956; Shubik and Ritchie, 1953; Eschenbrenner and Miller, 1945a,b). The pattern of tissue distribution of chloroform in mice also depends on mode of exposure. Cohen and Hood (1969), after chloroform inhalation, found highest levels in body fat (Table 4-6), while Brown et al. (1974) and Taylor et al. (1974) observed lower levels in fat and highest levels in liver and kidney following oral dosing (Table 4-7). The high liver levels of chloroform after oral administration may be due, in part, to first passage and extraction by the liver after this route of administration, to differences of time after exposure (2 versus 5 hours), and to metabolism and covalent binding of metabolites to cellular macromolecules (see below).

The sex difference in tissue distribution and binding of chloroform (and metabolites) in kidney and liver, noted by Brown and his colleagues (Taylor et al., 1974), appeared to be peculiar to mice. These workers did not observe such differences in male and female rats or squirrel monkeys (Brown et al., 1974).

4.4. EXCRETION

Elimination of chloroform from the body is perforce the sum of metabolism and excretion of unchanged chloroform via pulmonary and other routes. Unmetabolized chloroform is excreted almost exclusively through the lungs; however, metabolism of chloroform is extensive, with the proportion excreted unchanged dependent on body dose. Surprisingly, considering its historical importance, its longtime use as an industrial chemical and anesthetic agent, few controlled experimental studies in man have been made on the kinetics of excretion of chloroform.

4.4.1. Pulmonary Excretion

Figure 4-3 shows the time-course of pulmonary elimination of chloroform after accidental inhalation exposure to a mixture of solvents, including chloroform, carbon tetrachloride, trichloroethylene, and perchloroethylene. Stewart et al. (1965) determined, post-exposure, the alveolar air concentrations of these solvents by infrared and gas-liquid chromatography analysis. The kinetics of pulmonary excretion of the solvents are independent of one another. However, all, including chloroform, demonstrate the typical kinetics of gaseous vapor pulmonary elimination, that has been

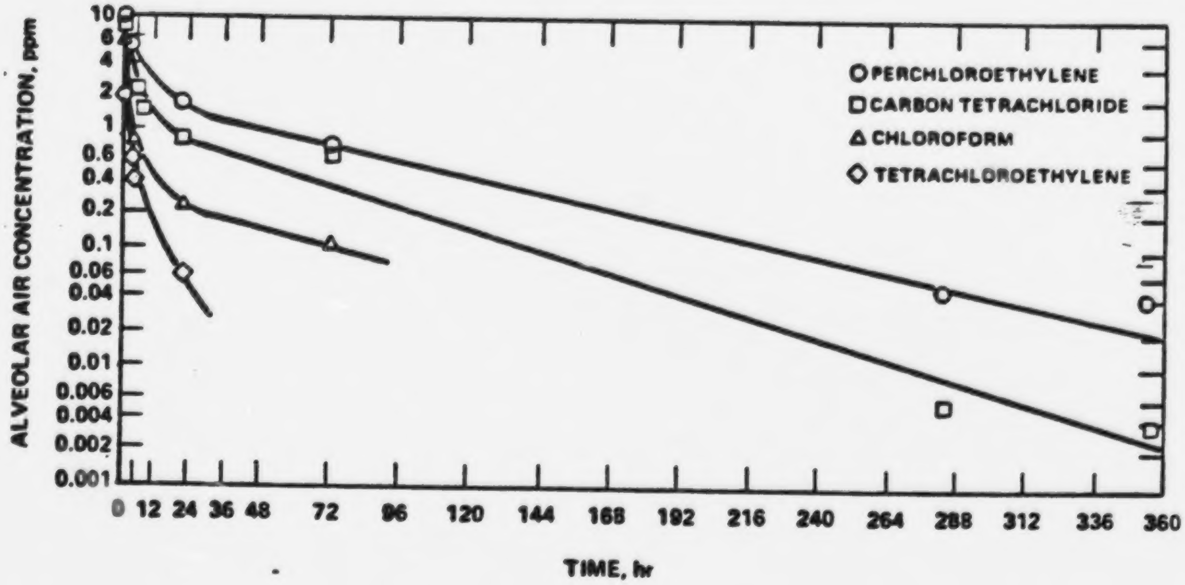

Figure 4-3. Exponential decay of chloroform, carbon tetrachloride, perchloroethylene and trichloroethylene in exhaled breath of 48 year-old male accidentially exposed to vapors of these solvents. The alveolar air (end tidal) was collected in a 50-mℓ glass pipette, and the sample was analyzed by infrared spectroscopy and gas-liquid chromatography. Initial values (30 min. post exposure) were chloroform, 7 ppm; carbon tetrachloride, 9.5 ppm; perchloroethylene, 11 ppm; and trichloroethylene, 4 ppm.

Source: Stewart et al. (1965).

observed experimentally for relatively hydrophobic, volatile gaseous anesthetics and industrial solvents (Eger, 1963; Fiserova-Bergerova et al., 1974, 1979, 1980; Droz et al., 1977). At termination of exposure with zero concentration in inspired air, chloroform (and the other solvents) immediately begins to be eliminated from the body into the lungs, with blood and alveolar air concentrations describing parallel exponential decay curves with three major components (Figure 4-3). These exponential components have been related by many investigators (Eger, 1963; Fiserova-Bergerova et al., 1978, 1979, 1980; Droz et al., 1977) to first-order kinetics of pulmonary elimination associated with desaturation of physiological compartments in accordance with a blood flow-limited model in which the rate constants are determined predominately by tissue perfusion, volume of tissue distribution and by partition coefficients:

$$\begin{array}{l}\text{Tissue uptake and}\\ \text{desaturation of}\\ \text{compartment,T}\end{array} = F_T \cdot \lambda_{bl/air} \exp\left(- \frac{F_T}{V_T \, \lambda_{T/bl}} \cdot t \right)$$

where F is blood flow through tissue compartment, V is volume of the compartment, g is partition coefficient, and exp is base of natural logarithm.

$$t_{1/2} = \frac{0.693 \, V \, \lambda_{T/bl}}{F}$$

Since three exponential components are typically observed experimentally, three physiological tissue compartments are included in the model described by a three-term exponential function of the form:

$$\text{Uptake, Desaturation} = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$$

where A, B, C are macrocoefficients and α, β, λ are hybrid constants (defined above). These terms represent three flow-limited major body compartments: (1) a vessel-rich group of tissues (VRG) with high blood flow and high diffusion rate constant (VRG: brain, heart, kidneys, liver and endocrine and digestive systems), (2) lean body mass (MG: muscle and skin), and (3) adipose tissue (FG). More recently, Fiserova-Bergerova and coworkers (1974, 1979, 1980) have mathematically reformulated this physiological, first-order model to accommodate the effect of metabolism on uptake, distribution and clearance of inhaled vapor compounds.

The half-times (t 1/2) of elimination from the physiological compartments (VRG < MG < FG) are independent of the body dose, but are dependent on tissue/blood partition coefficients and blood/air partition coefficients. Since these solvent compounds have high solubility in body fat (Table 4-1), they are eliminated slowly from fat depots with a long half-time of elimination, as illustrated by Stewart's patient (Stewart et al., 1965) in Figure 4-3. From Figure 4-3, it can be estimated graphically that chloroform has a half-time of elimination from the fat compartment (FG) of ≃36 hours, with similar long half-times for the highly fat soluble compounds, perchloroethylene and carbon tetrachloride.

There is limited information available for the half-times of pulmonary elimination of chloroform from the VRG and MG. From the early data of Lehmann and Hasegawa (1910) given in Table 4-3, the half-time of pulmonary elimination from the VRG appears to be ≃30 minutes. A similar estimate can be made from the data of Smith et al. (1973) and Morris (1951) at termination of anesthesia in man; these workers determined that blood chloroform concentration rapidly fell exponentially from 7 to 3.5 mg/dl within 30 minutes (Figure 4-2).

Pulmonary elimination of chloroform was investigated by Fry et al. (1972) in male and female volunteers given ^{13}C-chloroform in olive oil orally (by gelatin capsule). Chloroform was determined in expired air by GLC. Their data, summarized in Table 4-8, show that the amount of chloroform excreted through the lungs within 8 hours (expressed as a percentage of the dose, 0.1 to 1.0 g), increased (0 to 65%) in proportion to the dose. Following a peak blood concentration (0.5 mg/dl for a 500 mg dose) 1 hour after oral dosage, absorption, and distribution, the blood chloroform concentration declined exponentially with three components: (1) a very rapid disappearance, with half-time of 14 minutes possibly corresponding to VRG compartment kinetics, (2) a slower disappearance, with half-time of 90 minutes corresponding to MG kinetics, and (3) a very slow disappearance, with very long half-time from adipose tissue. This half-time was undetermined, but chloroform was detected in blood and breath 24 hours later. Fry and coworkers (1972) noted a linear relationship for their subjects between pulmonary excretion of chloroform and body weight deviation from ideal, an index of excessive leanness or excessive body fat from normal. Their data in Figure 4-4 show that for both male and female subjects given a standard oral dose of chloroform, lean subjects eliminate via the lungs a greater percentage of the dose, while overweight subjects eliminate less chloroform. The different slopes of the linear relationship for men and women presumably reflect the different proportion of adipose tissue in the two sexes. The bodies of women tend to contain higher proportions of fat than those of men (Geigy Scientific Tables, 1973). These observations reinforce the role of adipose tissue as a storage site for chloroform.

Brown and his coworkers (Brown et al., 1974; Taylor et al., 1974) have demonstrated an animal species difference in the amount of pulmonary

TABLE 4-8. PULMONARY EXCRETION OF $^{13}CHCl_3$ FOLLOWING ORAL DOSE (PERCENT OF DOSE)[a]

Subjects	Dose, g	Mean for 8 hours[b]	Range
8 M and F	0.5	40.3	17.8 to 66.6
1	1.0	64.7	NA
1	0.25	12.4	NA
1	0.10	nil	NA

Pulmonary excretion of $^{13}CO_2$ following 0.5 g oral dose of $^{13}CHCl_3$, Cumulative percent of dose[b]

	Time after dose, hr				
Subjects	0.5	1.75	2.5	5.5	7.5
Male (1)	2.1	24.1	35.9	49.2	50.6
Female (62.7 kg) (1)	0.5	10.7	28.3	47.5	48.5

[a]Recalculated from the data of Fry et al., 1972.
[b]Within 4% of value calculated for infinite time.
NA = not applicable.

excretion of ^{14}C-chloroform from a standard oral dose (60 mg/kg body weight) given in olive oil. Mice (three strains), rats, and squirrel monkeys excrete chloroform via the lungs (6, 20, and 79%, respectively, of the standard dose). This species difference is primarily related to the capacity to metabolize chloroform rather than differences in pulmonary kinetics, since,

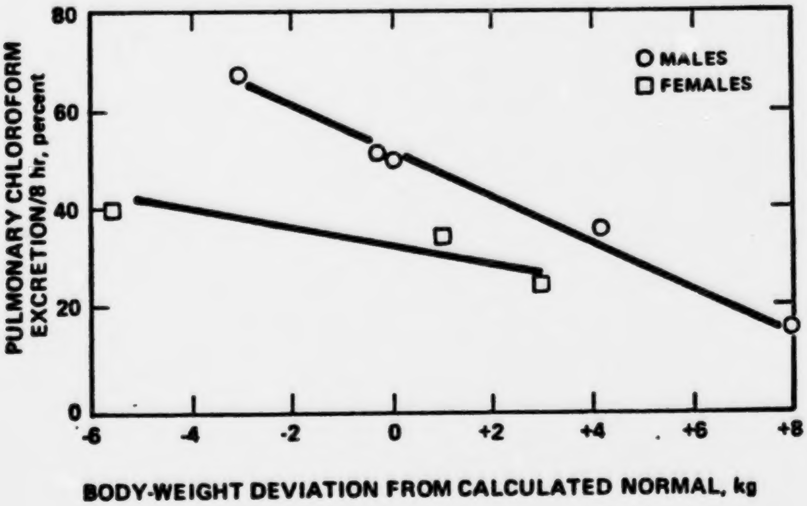

Figure 4-4. Relationship between total 8-hour pulmonary excretion of chloroform following 0.5-g oral dose in man and the deviation of body weight from ideal. The different slopes of the linear relationship for men and women reflect the different proportion of adipose tissue in the two sexes.

as shown in Table 4-9, the percentage of the dose metabolized to $^{14}CO_2$ is inversely proportional to that of pulmonary excretion. The mice, 48 hours after dosing, retained only 2% of chloroform radioactivity (Table 4-9).

Withey and Collins (1980) determined the kinetics of distribution and elimination of chloroform from blood of Wistar rats after intravenous administration of 3, 6, 9, 12 or 15 m/kg of chloroform given in 1 ml water intrajugularly. For all doses, the blood decay curves exhibited three components of exponential disappearance of chloroform (α, β, λ components) and "best" fitted a first-order three compartment model. Table 4-10 summarized the values obtained for the kinetic parameters. For volatile, lipophilic compounds, for which a major route of elimination is pulmonary, experiments utilizing dose administration via relatively large intravenous bolus injections (relative to rat total blood volume), and which measures only blood chloroform disappearance, provide a number of problems for data interpretation of elimination (pulmonary and metabolism) and/or tissue distribution. In these experiments, pulmonary elimination, which is rapid for organic solvents, occurs simultaneously with distribution and metabolism; in contrast, experiments in which the animal is preloaded by oral or inhalation administration, distribution is more readily separable from pulmonary elimination. After intravenous administration (Table 4-10), the rate constant k_e (for elimination of chloroform from the central compartment blood out of the body (principally via pulmonary excretion and/or metabolism) was dose-dependent and consistent with a half-time of elimination of only 3.6 min for the lowest dose and only 6.2 min for the highest dose. Since the half-times for distribution into other tissue compartments from the central compartment blood have longer half-times, it is likely that, of the dose introduced into the blood, a major portion (depending on dose) was excreted

TABLE 4-9. SPECIES DIFFERENCE IN THE METABOLISM OF ^{14}C-CHLOROFORM (ORAL DOSE OF 60 mg/kg)[a]

		^{14}C-radioactivity 48 hours after dose, mean values as percent dose				
Species	No.	Expired $^{14}CHCl_3$ or metabolites	Expired $^{14}CO_2$	Urine + feces	Carcass	Total
Mice CF/LP, CBA,C57 strains	19	6.1	85.1	2.6	1.8	95.6
Rats S-D	6	19.7	65.9	7.6	NR	93.2
Squirrel monkeys	6	78.7	17.6	2.0	NR	98.3

[a] Recalculated from the data of Brown et al., 1974.
NR = not recorded.

within a few minutes by the lungs. Further indication that the dose and/or mode of administration influenced the distribution and elimination of chloroform was shown by the proportional increase of the apparent volume of distribution, V_d (45 ml; 3 mg/kg to 89 ml; 15 mg/kg) and the decrease with dose in values for rate constants of transfer from blood to other tissue compartments. The volume of distribution (V_d) of chloroform was 89 ml for the highest dose or about 22% b.w., surprisingly low for a lipid soluble compound that is known to diffuse into all the major organ systems (Tables 4-5 and 4-6). Adipose tissue is known to be a major tissue compartment for chloroform [Section 4.3]; the clearance of chloroform from perirenal fat was found to be slow with a half-time of 106 min, and since the

TABLE 4-10. KINETIC PARAMETERS FOR CHLOROFORM AFTER I.V. ADMINISTRATION TO RATS (min -1 ± S.E.)

Dose,[a] mg/kg	V_d,[b] ml	α	β	γ	k_e	k_{12}	k_{21}	k_{13}	k_{31}
3.0	45.07 ±0.04	0.72 ±0.11	0.135 ±0.001	0.0287 ±0.0064	0.1907 ±0.0239	0.2346 ±0.0651	0.2575 ±0.0316	0.0921 ±0.0008	0.0421 ±0.0098
6.0	53.57 ±12.61	0.64 ±0.13	0.081 ±0.01	0.0158 ±0.0019	0.1874 ±0.0356	0.2681 ±0.0631	0.1862 ±0.0188	0.0730 ±0.0246	0.0233 ±0.0032
9.0	64.46 ±14.26	0.64 ±0.084	0.095 ±0.0001	0.0189 ±0.0009	0.1284 ±0.0217	0.2529 ±0.0489	0.2421 ±0.0064	0.0594 ±0.0034	0.0281 ±0.0042
12.0	80.62 ±20.20	0.32 ±0.20	0.060 ±0.028	0.0074 ±0.0056	0.1035 ±0.0232	0.1071 ±0.0774	0.1192 ±0.0676	0.0395 ±0.0253	0.0106 ±0.0084
15.0	89.13 ±12.83	0.35 ±0.048	0.070 ±0.006	0.0134 ±0.0005	0.1124 ±0.0110	0.1104 ±0.0256	0.1523 ±0.0188	0.0396 ±0.0104	0.0193 ±0.0018
15.0; $t_{\frac{1}{2}}$ min		2.1	9.9	51.7	6.2	6.3	4.6	17.5	35.9

[a] 2 to 4 rats/dose.
[b] V_d = volume of distribution.

SOURCE: Withey et al., 1982.

rate constant, k_{31} was given as 0.0193 min^{-1} (for 15 mg/kg dose) indicating a half-time of 36 min, the adipose tissue appears to be a deep compartment. These investigators believe that their kinetic data show no evidence in the rat of nonlinear or dose-dependent Michaelis-Menten kinetics and they suggest that a dose of 15 mg/kg is below hepatic metabolism saturation.

However, Reynolds et al. (1984a,b) have observed dose-dependent metabolism and first-pass effect in rats given 12 or 36 mg/kg ^{14}C-chloroform by gavage in mineral oil. For these two oral doses, 5% and 12%, respectively, were excreted unchanged in exhaled air with peak rates of 0.71 and 1.81 mg hr^{-1} kg^{-1} at 15 and 30 min, respectively, after dosing. The percentages of the two doses metabolized (to $^{14}CO_2$ collected in exhaled air) was 67% and 68%, respectively, at peak rate of 4.4 and 5.5 mg hr^{-1} kg^{-1}, 30-45 and 60-105 min after ministration. These investigators fitted the pulmonary elimination data to a linear two-compartment model and determined the apparent half-times of absorption, distribution, and pulmonary elimination for the 12 and 36 mg/kg gavage doses, respectively; for chloroform absorption, 0.08 and 0.13 hr, distribution 0.29 and 0.41 hr, pulmonary elimination 3.83 and 2.27 hr; and for pulmonary elimination of CO_2 metabolite, 2.1 and 5.6 hr. These dose-dependent half-times indicate strongly a dose-dependent disposition of chloroform in the rat in accordance with Michaelis-Menten kinetics for metabolism.

4.4.2. Other Routes of Excretion

Chloroform is not eliminated in significant amounts from the body by any route other than pulmonary. Studies of chlorinated compounds in the urine after chloroform inhalation exposure or peroral dosage to animals and humans have failed to detect unchanged chloroform (Brown et al., 1974; Fry et al., 1972). Brown et al. (1974) identified chloroform in high concentration in the bile of squirrel monkeys after oral ^{14}C-chloroform dosage, and suggested an active enterohepatic circulation in this species. For monkeys, they found only 2% of dose radioactivity in combined urine and feces collected for 48 hours after dosing (Table 4-9), and only 8 and 3% for rats and mice, respectively.

4.4.3. Adipose Tissue Storage

There is no definitive experimental evidence in the literature concerning bioaccumulation after chronic or repeated daily exposure to chloroform. However, there are practical reasons to believe that extended residence in body fat occurs. In man, chloroform has a relatively high fat tissue/blood partition coefficient of 35 (Table 4-2), a long half-time of elimination from adipose tissue compartments of ≃36 hours (Figure 4-3), and it has been detected in blood and breath 24 to 72 hours after a single exposure (Stewart, 1974; Fry et al., 1972; Stewart et al., 1965). Figure 4-5 clearly shows the slow elimination of chloroform from the adipose tissue of dogs following a 3-hour anesthesia (Chenoweth et al., 1962). Despite the rapid exponential decline of blood levels of chloroform within 3 hours after termination of anesthesia, significant levels of chloroform were still present 20 hours later.

Fry et al. (1972) have provided indirect evidence in man of the storage of chloroform in body fat (Figure 4-4), while analysis of body fat of animals given a single inhalation exposure or oral dosage demonstrated marked accumulation of chloroform in this tissue (Taylor et al., 1974; Cohen and Hood, 1969) (Tables 4-5, 4-6, 4-7). Of direct relevance to man are the observations of two research groups: McConnell et al. (1975), who demonstrated the occurrence of significant amounts of chloroform (and other chlorinated hydrocarbons) in autopsy tissues (highest concentrations in body

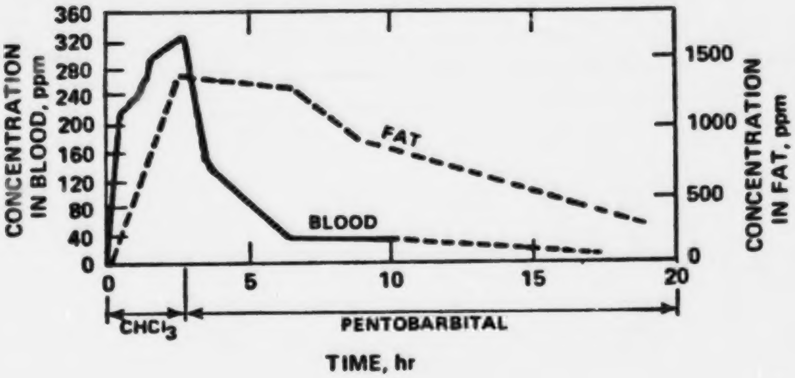

Figure 4-5. Blood and adipose tissue concentrations of chloroform during and after anesthesia in a dog. Note the high prolonged levels of chloroform in adipose tissue (broken line) for 20 hours even after rapid exponential fall in blood concentration (solid line) with termination of chloroform anesthesia after three hours.

Source: Chenoweth et al. (1962).

fat) of humans exposed only to ordinary ambient air (Table 4-4); and of Conkle et al. (1975), who analyzed the GC-MS alveolar air of eight fasting healthy men working in a nonindustrial environment and found in three men significant rates of pulmonary excretion of chloroform (Table 4-11), as well as other halocarbons (for example, methylene chloride, dichlorobenzene, methylchloroform) in all eight men.

4.5. BIOTRANSFORMATION OF CHLOROFORM

4.5.1. Known Metabolites

The haloforms, and chloroform in particular, long have been known to undergo extensive mammalian biotransformation. Zeller (1883) demonstrated an increased daily urinary inorganic chloride excretion representing 25 to 60% of the dose in dogs given oral doses of chloroform (7 to 10 g in gelatin capsules). Eighty years later, Van Dyke et al. (1964), using

TABLE 4-11. LEVELS OF CHLOROFORM IN BREATH OF FASTED NORMAL HEALTHY MEN

Subject	Age	Chloroform excretion, μg/hr
A	34	2.0
B	28	ND
C	33	ND
D	38	ND
E	47	11.0
F	28	ND
G	38	ND
H	23	0.22

ND = not detected.

SOURCE: Conkle et al., 1975.

^{36}Cl-chloroform, confirmed in rats that the extra urinary inorganic chloride originated from the metabolism of chloroform. Zeller (1883) also found, in the urine of his dogs given chloroform, a levo-rotatory oxidative metabolite that he suggested to be the glucuronide of trichloromethanol, a compound only recently postulated as an intermediate of P_{450} oxidative metabolism (Figure 4-6). Van Dyke and coworkers (1964) also found evidence for ^{36}C-metabolites (≃2% dose) in the urine of their rats given chloroform. However, other investigators (Brown et al., 1974; Fry et al., 1972; Paul and Rubinstein, 1963; Butler, 1961) with newer methodologies have not been able to identify lesser chloromethanes in the urine or breath of mouse, rat, or man after chloroform exposure.

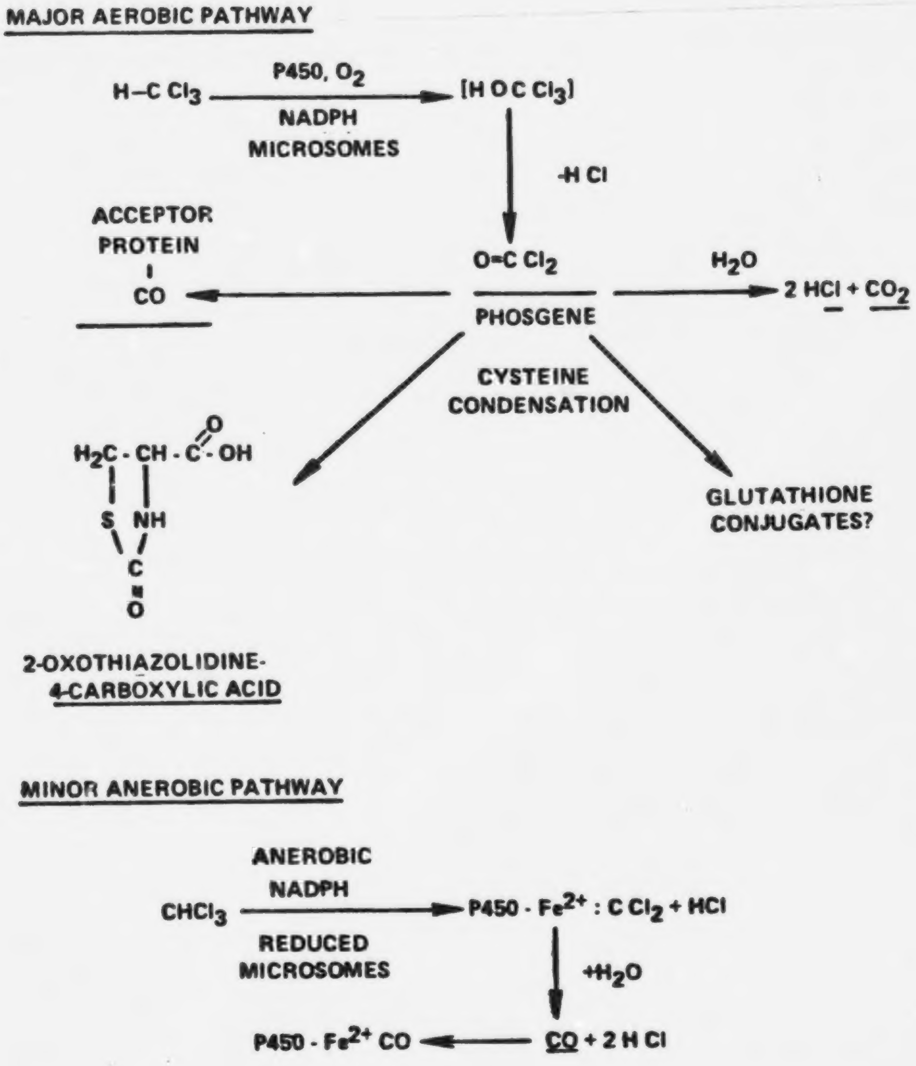
MAJOR AEROBIC PATHWAY
H–C Cl3
P450, O2
NADPH
MICROSOMES
[H O C Cl3]
-H Cl
ACCEPTOR
PROTEIN
CO
O=C Cl2
PHOSGENE
H2O
2 HCl + CO2
CYSTEINE
CONDENSATION
H2C - CH - C - OH
O
S
NH
C
O
2-OXOTHIAZOLIDINE-
4-CARBOXYLIC ACID
GLUTATHIONE
CONJUGATES?
MINOR ANEROBIC PATHWAY
CHCl3
ANEROBIC
NADPH
REDUCED
MICROSOMES
P450 · Fe2+ : C Cl2 + HCl
+H2O
CO + 2 H Cl
P450 · Fe2+ CO

Figure 4-6. Metabolic pathways of chloroform biotransformation. (Identified CH Cl_3 metabolites are underlined.)

In addition to the chloride ion, it has been established from both *in vivo* and *in vitro* studies that the major end-product metabolite of chloroform is carbon dioxide (CO_2) (Brown et al., 1974; Fry et al., 1972; Rubinstein and Kanics, 1964; Van Dyke et al., 1964; Paul and Rubinstein, 1963), with phosgene identified as the immediate precursor metabolite from *in vitro* studies (Mansuy et al., 1977; Pohl et al., 1977; Ilett et al., 1973) (Figure 4-6). CO_2 from chloroform metabolism is primarily excreted through the lungs, but a small percentage (<7%) is incorporated into endogenous metabolites and excreted into the urine as bicarbonate, urea, methionine, and other amino acids (Brown et al., 1974). Carbon monoxide (CO) has also been identified as a very minor metabolite of anaerobic chloroform metabolism (Figure 4-6), both from *in vitro* studies (Ahmed et al., 1977; Wolf et al., 1977) and *in vivo* animal studies (Anders et al., 1978; Bellar et al., 1974).

In addition to chloroform metabolites that are excreted, phosgene and other "reactive intermediates" of chloroform metabolism interact with and covalently bind to tissue acceptors such as protein and lipids (Docks and Krisna, 1976; Uehleke and Werner, 1975; Brown et al., 1974; Ilett et al., 1973; Cohen and Hood, 1969; Reynolds, 1967; Cessi et al., 1966).

The liver is the principal site of chloroform metabolism, although Paul and Rubinstein (1963) and Butler (1961) found that rat kidney, adipose tissue, and skeletal muscle also converted chloroform to CO_2 (25, 0.8, and 8% of liver, respectively).

Recently it has been shown that chloroform is metabolized by the kidney via metabolic pathways similar to those in the liver, although to a lesser extent (per unit weight of renal cortical tissue) (Smith and Hook, 1983, 1984; Pohl et al., 1984; Branchflower et al., 1984).

4.5.2. Magnitude of Chloroform Metabolism

Chloroform is metabolized to differing extents in man and other animal species. Since chloroform and other halogenated hydrocarbons are thought to produce pathological effects by metabolism in target tissues to reactive intermediates that covalently bind to macromolecules (Chapter 5), the total capacity to metabolize chloroform as well as individual tissue sites of metabolism are important determinants of expected interspecies differences in toxic susceptibility. This interdependence between intensity of toxic response and metabolism, and interspecies differences in magnitude of metabolism, are important considerations in extrapolation from experimental animal to man (Reitz et al., 1978).

Few studies have been made of the capacity of man to metabolize chloroform; virtually no studies have been made of the pharmacokinetic, endocrine, genetic, and environmental factors modifying metabolism in man. The early studies of Lehmann and Hasegawa (1910) on the retention of chloroform from inspired air inhaled by three volunteers (4500 to 5000 ppm, average of 64%) (Table 4-3) suggest that 36% of pulmonary uptake of chloroform in man is metabolized. Similarly, a retention value of 67%, calculated from the data of Smith et al. (1973) for patients inhaling 10,000 ppm chloroform during surgical anesthesia (Figure 4-2), indicates 33% chloroform metabolism during inhalation exposure. A similar estimate of the extent of chloroform metabolism during anesthesia has been made by Feingold and Holaday (1977). These workers simulated, with a computer, chloroform inhalation kinetics using a nonlinear whole-body compartmental model, and found that the percent of chloroform uptake metabolized was 30%. This rate of metabolism remained constant during 8 hours of anesthesia, and continued for several days following termination of anesthesia, presumably from chloroform stored during anesthesia in adipose tissue.

Fry et al. (1972) have investigated the metabolism of chloroform in man after single oral doses. Isotopically labeled ^{13}C-chloroform dissolved in 1.0 ml olive oil/gelatin capsule was given to 12 healthy male and female volunteers (58 to 60 kg body weight) at doses of 0.1 to 1.0 g. For two of the volunteers, pulmonary excretion of $^{13}CO_2$ in expired air from the metabolism of chloroform was serially connected over a 7.5 hour period and analyzed by mass spectrometry. The results given in Table 4-8 show that 49 and 51% of a 0.5 g dose was metabolized to $^{13}CO_2$. Pulmonary excretion of unchanged ^{13}C-chloroform during a comparable period (8 hours) in a separate experiment with these two subjects were 67 and 40%, respectively. No metabolites other than CO_2 (e.g., methylene dichloride, tetrachloroethane) were found in expired air, and chloroform was not found in the urine. These results indicate that (1) virtually all of an oral chloroform dose (0.5 g) can be accounted for by pulmonary excretion of CO_2 and unchanged chloroform; (2) metabolism of chloroform to CO_2 is $\approx$50% of this dose; and (3) absorption and metabolism are rapid and virtually complete within 5 hours as shown in Table 4-8, possibly because of first pass through the liver. In this respect, the kinetics of metabolism of oral doses may differ substantially from inhalation doses. Furthermore, the data of Table 4-8 suggest that the fraction of the dose metabolized is dose-dependent. Thus, an oral dose of 0.1 g was completely metabolized (100%), with no chloroform excreted unchanged through the lungs; but for a 1.0 g dose, 65% was excreted and only 35% metabolized. These results suggest that metabolism is rate-limited in man, since a diminishing proportion of dose is metabolized with increasing dose (Table 4-8).

In man, Chiou (1975) has shown that up to 38% of an oral dose of chloroform is metabolized in the liver, and up to about 17% is excreted intact from the lungs before the chloroform reaches the systemic circulation, an example of a first-pass effect.

Animal experiments demonstrate a marked species difference in the metabolism of chloroform. Early experiments in the 1960s by Paul and Rubinstein (1963), Van Dyke et al. (1964), and Cohen and Hood (1969) with mice and rats given ^{14}C-chloroform indicated a minimal metabolism of chloroform ($\approx 4\%$) occurred in these species. More recent studies by Brown and his coworkers (Brown et al., 1974; Taylor et al., 1974) have shown that mice and rats metabolize chloroform to CO_2 extensively (65 to 85%), and to a greater extent than non-human primates or man. These investigators gave equivalent oral doses (60 mg/kg) of ^{14}C-chloroform to mice (3 strains), rats, and squirrel monkeys, and determined ^{14}C-labeled chloroform or volatile metabolites and $^{14}CO_2$ in expired air, ^{14}C-radioactivity in urine, and ^{14}C-radioactivity remaining in animals at sacrifice 4 to 8 hours after dosing. Total recovery of ^{14}C-chloroform radioactivity was excellent and accounted for 93 to 98% of the administered dose. Their results are summarized in Table 4-9. Using $^{14}CO_2$ as a measure of the fraction of the chloroform dose metabolized (intermediate chloromethane metabolites were not found in breath or urine), mice metabolized 85% of the dose, rats, 66%, and squirrel monkeys, 28%. A further 2 to 8% of ^{14}C-radioactivity ($^{14}CO_2$ incorporated into urea, bicarbonate, and amino acids) were found in urine. They found no strain difference in mice, or sex difference in mice, rats, or monkeys in capacity to metabolize chloroform, or in tissue distribution and binding of metabolites, except for mice, where kidney radioactivity concentration was greater in males than females and lesser in livers of males than females (Table 4-7). In rats, also, with oral doses of 12 and 36 mg/kg chloroform, Reynolds et al. (1984a,b) found 67% and 69% of the dose, respectively, was metabolized to CO_2, indicating at least in the rat extensive, rate-limited (above 60 mg/kg) metabolism. These findings of Brown and coworkers of large interspecies differences for metabolism of chloroform and marked sex differences in mice (but not other species) for tissue distribution and covalent binding of intermediate metabolites to tissue macromolecules in liver and kidney emphasize the difficulties and dangers of extrapolating studies in lower animals to man (Reitz et al., 1978).

4.5.3. Enzymic Pathways of Biotransformation

The postulation has been made that a reactive metabolite of $CHCl_3$ is responsible for its liver and renal toxicity in man (von Oettingen, 1964; Conlon, 1963) and experimental animals (Bhooshan et al., 1977; Pohl et al., 1977; Ilett et al., 1973; Klaassen and Plaa, 1966), and possibly the production of liver tumors in mice (Eschenbrenner and Miller, 1945a) (see generally Docks and Krishna, 1976; Uehleke and Werner, 1975; Brown et al., 1974; Ilett et al., 1973; Reynolds, 1967; Paul and Rubinstein, 1963). For example, when rats or mice are treated with ^{14}C-chloroform, the extent of hepatic necrosis parallels the amount of ^{14}C-label bound irreversibly to liver protein (Docks and Krishna, 1976; Brown et al., 1974; Ilett et al., 1973). Both necrosis and binding are potentiated by pretreatment of animals with phenobarbital, a known inducer of liver microsomal metabolism, and inhibited by pretreatment with the inhibitor piperonyl butoxide. Chloroform administration also decreases the level of liver glutathione in rats pretreated with phenobarbital, further suggesting that a reactive metabolite is produced (Docks and Krishna, 1976; Brown et al., 1974). The results of *in vitro* studies with rat and mouse liver and kidney microsomes support the in vivo observations by establishing that ^{14}C-chloroform is metabolized to a reactive metabolite which binds covalently to microsomal protein (Bhooshan et al., 1977; Sipes et al., 1977; Uehleke and Werner, 1975; Ilett et al., 1973; Pohl et al., 1984; Branchflower et al., 1984; Smith and Hook, 1984). This metabolic process is oxygen dependent and appears to be mediated by cytochrome P_{450} which is inducible by phenobarbitol (Sipes et al., 1977; Uehleke and Werner, 1975; Ilett et al., 1973).

The demonstrations by Pohl et al. (1977) and Mansuy et al. (1977) of carbonyl chloride (phosgene) formation from chloroform by rat microsomal preparations suggest that phosgene may be the key causal agent for these toxic effects. The finding of Weinhouse and collaborators (Shah et al., 1979) that phosgene is also a reactive metabolic intermediate in the metabolism of carbon tetrachloride emphasizes basic similarities in the metabolism and toxicities of these two chloroalkanes. Figures 4-6 and 4-7 show for comparison the currently proposed pathways of metabolism of chloroform in liver and kidney and of carbon tetrachloride.

Figure 4-6 indicates that the initial step in the metabolism of chloroform involves the oxidation of the aliphatic carbon (H-C) to trichloromethanol by phenobarbital inducible cytochrome P_{450} (Sipes et al., 1977; Uehleke and Werner, 1975; Ilett et al., 1973). This metabolic step has been suggested by Mansuy et al. (1977) and Pohl et al. (1977) as the precursor of phosgene formed by rat microsomes in vitro from chloroform. Phosgene was confirmed as a metabolite by reaction with cysteine to give 2-oxothiazolidine-4-carboxylic acid which was identified by GC-CIMS. Trichloromethanol is highly unstable and spontaneously dehydrochlorinates to produce phosgene (Seppelt, 1977). The electrophilic phosgene reacts with water to yield CO_2, a known metabolite of $CHCl_3$ in vitro (Rubinstein and

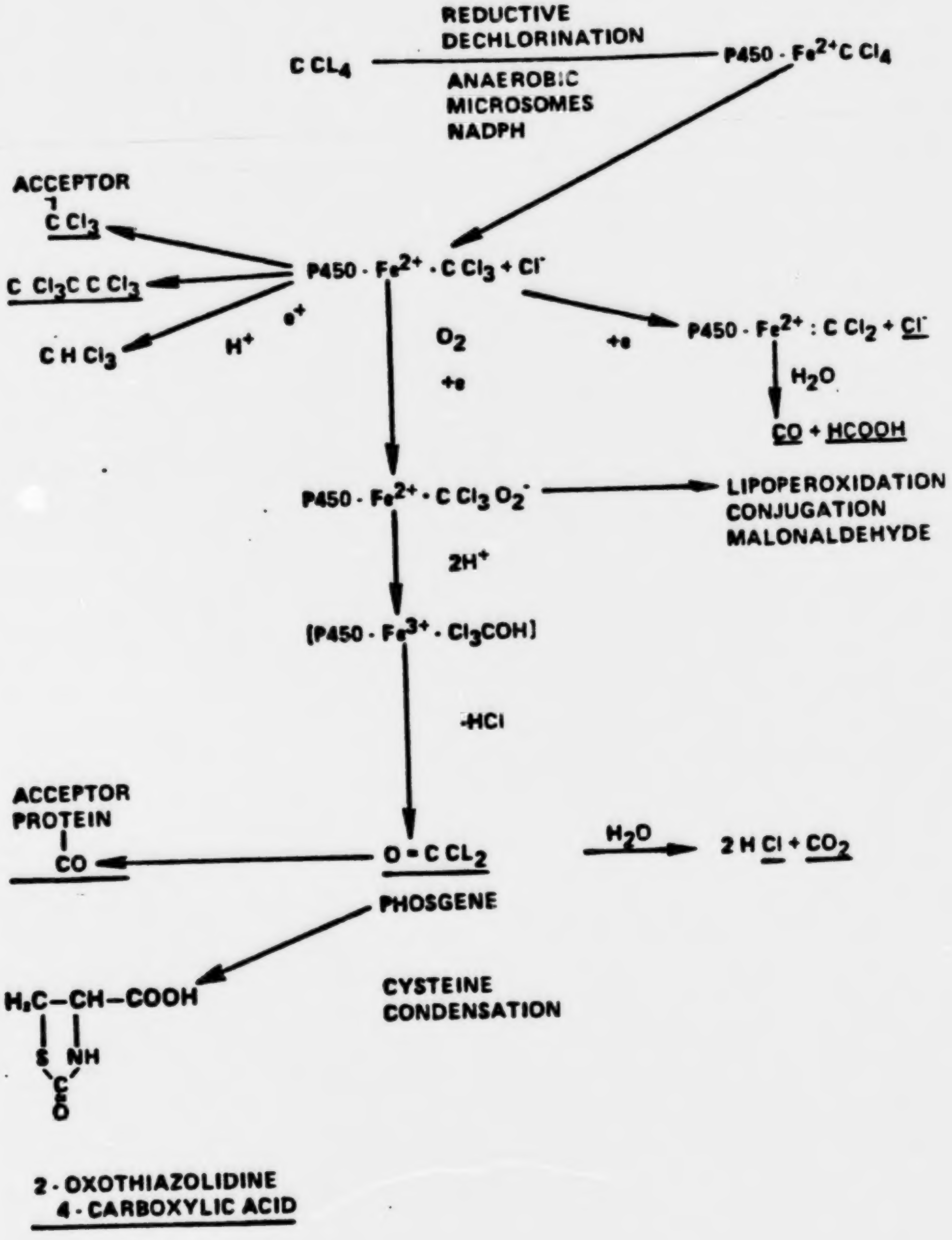
REDUCTIVE DECHLORINATION
C CL4
P450 · Fe2+C Cl4
ANAEROBIC MICROSOMES NADPH
ACCEPTOR
C Cl3
C Cl3C C Cl3
C H Cl3
H+
e+
P450 · Fe2+ · C Cl3 + Cl-
O2
+e
+e
P450 · Fe2+ : C Cl2 + Cl-
H2O
CO + HCOOH
P450 · Fe2+ · C Cl3 O2-
LIPOPEROXIDATION
CONJUGATION
MALONALDEHYDE
2H+
[P450 · Fe3+ · Cl3COH]
-HCl
ACCEPTOR PROTEIN
CO
O = C CL2
PHOSGENE
H2O
2 H Cl + CO2
H2C–CH–COOH
S
NH
C
O
CYSTEINE CONDENSATION
2 · OXOTHIAZOLIDINE 4 · CARBOXYLIC ACID

Figure 4-7. Metabolic pathways of carbon tetrachloride biotransformation. ($C\ Cl_4$ metabolites identified are underlined).

Source: Shah et al. (1979).

Kanics, 1964; Paul and Rubinstein, 1963) and in vivo (Brown et al., 1974; Fry et al., 1972), with protein to form a covalently bound product (Pohl et al., 1977; Sipes et al., 1977; Uehleke and Werner, 1975; Brown et al., 1974; Ilett et al., 1973), or with cysteine (Pohl et al., 1977), and possibly with glutathione (Docks and Krishna, 1976; Brown et al., 1974). The finding that deuterated chloroform ($CDCl_3$) depletes glutathione in the livers of rats less than $CHCl_3$ supports this notion (Docks and Krishna, 1976).

The postulated oxidation of the C-H bond of chloroform by P_{450} to produce trichloromethanol which spontaneously yields phosgene is further supported by the observations of Pohl and Krishna (1978). These workers found that chloroform metabolism to phosgene by rat liver microsomes is oxygen and NADPH dependent, and inhibited by CO and SKF 525-A. Moreover, in the presence of cysteine and $^{18}O_2$ atmosphere, $^{18}O_2$ is incorporated into the 2-oxo position of 2-oxothiazolidine-4-carboxylic acid. Oxidative cleavage of the C-H bond appears to be the rate-determining step, since deuterium labeled chloroform ($CDCl_3$) is biotransformed into phosgene slower than $CHCl_3$; $CDCl_3$ appears also to be less hepatotoxic than $CHCl_3$. Pohl (1980) has further characterized the metabolism of chloroform in rat liver microsomes by measuring the covalent binding of $^{14}CHCl_3$ and C^3HCl_3 to microsomal protein. Chloroform does not appear to be activated by reductive dechlorination to the radical $\cdot CHCl_2$, because the ^3H-label does not bind to microsome protein as does the ^{14}C-label.

Figure 4-7 summarizes current knowledge of the biotransformation of carbon tetrachloride. The first step is a rapid reductive formation of the trichloromethyl ($\cdot CCl_3$) radical by complexing with one or more of the P_{450} cytochromes (Shah et al., 1979; Poyer et al., 1978; Recknagel and Glende, 1973). This radical undergoes several reactions in addition to binding to lipids (Villarruel and Castro, 1975; Uehleke and Werner, 1975; Villarruel et al., 1975; Gordis, 1969; Reynolds, 1967) and protein (Uehleke et al., 1977; Uehleke and Werner, 1973), although not to nucleic acids (Uehleke et al., 1977; Uehleke and Werner, 1975; Reynolds, 1967). Anaerobically, the addition of a proton and electron yields chloroform (Glende et al., 1976; Uehleke et al., 1973; Fowler, 1969; Butler, 1961), dimerization to hexachloroethane (Uehleke et al., 1973; Fowler, 1969), or further reductive dechlorination to CO via the carbene CCl_2 (Wolf et al., 1977). Aerobically, the $\cdot CCl_3$ radical is oxidized by the P_{450} system to trichloromethanol (Cl_3COH), which is the precursor of phosgene (Cl_2CO) that Weinhouse and colleagues (Shah et al., 1979) have shown to be an intermediate in carbon tetrachloride metabolism by rat liver homogenates. Hydrolytic dechlorination of phosgene yields CO_2 (Shah et al., 1979).

Under normal physiological conditions (i.e., aerobic conditions), a minimal formation of chloroform might be expected to occur. Carbon tetrachloride yields a chloroform most readily *in vitro* under anaerobic conditions and its formation is inhibited by oxygen (Uehleke et al., 1977; Glende et al., 1976). Shah et al. (1979) observed that chloroform does not compete successfully with carbon tetrachloride for initial binding to P_{450} cytochrome (Sipes et al., 1977; Recknagel and Glende, 1973). Wolf et al. (1977) also found that binding of chloroform to reduced cytochrome P_{450} was very slow compared to that of carbon tetrachloride.

Anders and coworkers (Anders et al., 1978; Ahmed et al., 1977) have shown that dihalomethanes and trihalomethanes, including chloroform, also yield CO as a metabolite. Intraperitoneal administration of haloforms (1 to 4 mmoles/kg) to rats led to dose-dependent elevations in blood CO levels. Treatment of the rats with either phenobarbital (but not 3-methylcholanthrene) or SKF 525-A, respectively, increased or decreased metabolism to CO. The order of yield of CO from the iodoforms was greatest for iodoform > bromoform > chloroform for the same dose. Thus, chloroform was minimally metabolized to CO (i.e., to less than one-tenth of that for iodoform or bromoform). Similar findings were made by these workers with rat liver microsomes (Ahmed et al., 1977); they found that metabolism of the haloforms to CO by rat liver microsomes (1) required NADPH; (2) could proceed anaerobically but was increased 2-fold by O_2; (3) was increased by pretreatment with phenobarbital and inhibited by SKF 525-A or $COCl_2$ pretreatment; and (4) was stimulated by glutathione or cysteine addition in both anaerobic (3-fold) or aerobic (8-fold) conditions. These results suggested that haloforms were metabolized to CO via a cytochrome P_{450} dependent system. However, chloroform was a poor substrate compared to iodoform or bromoform, yielding <2% of its quantity of CO as formed from equimolar concentrations of these halomethanes. Wolf et al. (1977) also found that chloroform, to a very limited extent, was metabolized to CO by reduced rat P_{450} preparations. These workers investigated the spectral and biochemical interactions of a series of halogenated methanes with rat liver microsomes under anaerobic reducing conditions. Tetrahalogens (e.g., CCl_4) and trihalogens (e.g., $CHCl_3$) all formed complexes with reduced cytochrome P_{450} with absorption peak at 460 to 465. A shift to 454 occurred with CO formation and subsequent complexing of CO to P_{450}. CO formation required NADPH, was higher in microsomes from phenobarbital- and 3-methylcholanthrene-treated rats, and was not found at high oxygen concentrations (>8%). Testai and Vittozzi (1984) have recently reported similar observations. These investigators compared the metabolism of chloroform to protein covalent bound metabolites and complexing with cytochrome P_{450} by rat liver microsomes maintained at pH 7.4 under anaerobic (<1% pO_2) and aerobic (>6% pO_2) conditions with added NADPH and generator. Covalent binding to microsomal protein in the absence of molecular oxygen was reduced to 30% of that established with aerobic conditions, and a greater loss of functional cytochrome P_{450} was observed. Differential spectra obtained below a 6% pO_2 tension showed appearance of a maximum at about 450 nm. Since anaerobically formed metabolites were very effective in producing P_{450} loss, it was suggested that the spectral change was possibly due to the formation of a chloroform metabolite-adduct with cytochrome P_{450}.

Figure 4-8 shows the relative rates of CO formation from carbon tetrachloride and other polyhalomethanes. Chloroform, in comparison to carbon tetrachloride, was a very poor reaction substrate, and binding of chloroform to reduced cytochrome P_{450} was extremely slow compared to that of carbon tetrachloride. Wolf et al. (1977) proposed the reduction sequence shown in Figures 4-6 and 4-7 for the reductive dechlorination of chloroform and of carbon tetrachloride to yield CO via a carbene (CCl_2) intermediate. The physiological importance of this pathway of metabolism appears to be more significant for carbon tetrachloride than for chloroform.

4.6. COVALENT BINDING TO CELLULAR MACROMOLECULES

4.6.1. Proteins and Lipids

Reactive intermediates of the metabolism of chloroform (phosgene, carbene, $\cdot Cl$) and carbon tetrachloride ($\cdot CCl_3$, phosgene, carbene, $\cdot Cl$) that irreversibly bind to cellular macromolecules (covalent binding) are generally believed to result in an alteration of cellular integrity, which leads to centrolobular hepatic necrosis and renal proximal tubular epithelial damage. Chloroform, mole for mole, is generally accepted to be less hepatotoxic than

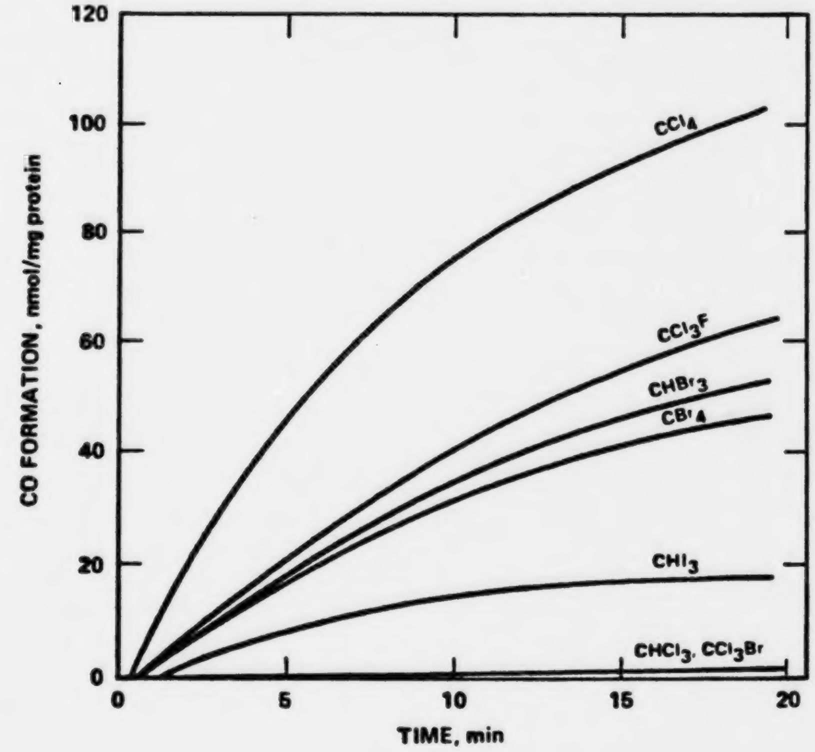

Figure 4-8. Rate of carbon monoxide formation after addition of various halomethanes to sodium dithionite-reduced liver microsomal preparations from phenobarbitol-treated rats. Note the low rate of metabolism of chloroform to CO compared to carbon tetrachloride.

Source: Wolf et al. (1977).

carbon tetrachloride (Brown, 1972; Klaassen and Plaa, 1969; Plaa et al., 1958).

Chloroform in non-lethal doses produces renal damage in mice, dogs, and man (Bhoosan et al., 1977; Pohl et al., 1977; Ilett et al., 1973; Klaassen and Plaa, 1966, 1967; Bennet and Whigham, 1964; von Oettingen, 1964; Conlon, 1963; Plaa et al., 1958; Culliford and Hewitt, 1957; Hewitt, 1956; Shubik and Ritchie, 1953); whereas in experimental animals, carbon tetrachloride does not do so (Storms, 1973; Klaassen and Plaa, 1966; Plaa and Larson, 1965; Bennet and Whigham, 1964; Culliford and Hewitt, 1957; Hewitt, 1956; Shubik and Ritchie, 1953), although it does in man (New et al., 1962; Guild et al., 1958). To explain these species differences in toxicity as well as known intraspecies (Hill et al., 1975; Deringer et al., 1953; Shubik and Ritchie, 1953) and sex differences (Taylor et al., 1974; Ilett et al., 1973; Bennet and Whigham, 1964; Culliford and Hewitt, 1957; Hewitt, 1956; Deringer et al., 1953; Shubik and Ritchie, 1953; Eschenbrenner and Miller, 1945b), prevailing concepts implicate (1) differences in the rates of metabolism and organ system capacities for metabolism, which in turn determine the amount of irreversible macromolecular binding and (2) differences in the enzyme pathways for metabolism of the two haloalkanes (Figures 4-6 and 4-7).

Carbon tetrachloride, by a reductive dechlorination via complexing with reduced P_{450}, yields the trichloromethyl free radical ($\cdot CCl_3$) (Recknagel and Glende, 1973; Slater, 1972) (Figure 4-7), which can covalently bind to lipid and protein (Shah et al., 1979; Villarruel et al., 1975; Castro and Diaz Gomez, 1972; Reynolds, 1967), and can also initiate peroxidation of polyenoic fatty acids (Slater, 1972; Recknagel and Ghoshal, 1966). Chloroform does not appear to be activated to free radicals ($\cdot CCl_3$ or $\cdot CHCl_3$), but does bind covalently to liver lipid and protein (Sipes et al., 1977; Docks and Krishna, 1976; Uehleke and Werner, 1975; Brown et al., 1974; Ilett et al., 1973), and initiates lipid peroxidation in some circumstances (Koch et al., 1974; Ilett, 1973; Brown, 1972; Slater, 1972). Several investigators have shown that diene conjugates (products of lipoperoxidation) are not increased *in vivo* in normal rats when chloroform is inhaled or injected intraperitoneally (Brown et al., 1974; Brown, 1972; Klaassen and Plaa, 1969), but only when rats are pretreated with phenobarbital and the metabolism of chloroform is greatly enhanced (Brown et al., 1974; Brown, 1972). Studies *in vitro* show diene conjugation and malonaldehyde formation (an index of lipoperoxidation) by microsomes of phenobarbital-pretreated rats were not increased but decreased by the addition of chloroform (Brown, 1972; Klaassen and Plaa, 1969), suggesting that with isolated microsomes, metabolism of chloroform is too small for sufficient quantities of reactive intermediates to accumulate and initiate lipoperoxidation. However, Rubinstein and Kanics (1964) found chloroform to be more rapidly metabolized by rat microsomal fractions than carbon tetrachloride (see also Table 4-12). These findings indicate that differences in metabolic activation [carbon tetrachloride to produce free radicals (Figure 4-7), but chloroform primarily to phosgene (Figure 4-6)] explain the greater potential of carbon tetrachloride for initiating lipoperoxidation. Table 4-12 shows the data of Uehleke et al. (Uehleke et al., 1977; Uehleke and Werner, 1975) for the covalent binding of rabbit microsomes following incubation with ^{14}C-labeled chloroform and carbon tetrachloride. Both protein and lipid binding of ^{14}C-radioactivity are 4-fold and 20-fold, respectively, more extensive for carbon tetrachloride than chloroform; lipids are labeled preferentially by carbon tetrachloride but are not by chloroform. Furthermore, covalent binding from chloroform metabolism occurs mainly with anaerobic conditions (a minor metabolic

TABLE 4-12. COVALENT BINDING OF RADIOACTIVITY FROM ^{14}C-CHLOROFORM AND ^{14}C-CARBON TETRACHLORIDE IN MICROSOMAL INCUBATION *IN VITRO* (nmol/mg in 60 min)

	Incubation condition	Microsomal Protein	Microsomal Lipid	To added serum albumin
$^{14}C-CCl_4$	N_2	20.0	76.0	1.4
$^{14}C-CHCl_3$	N_2	5.1	4.1	0.9
	O_2	8.5	7.0	1.7

[a]Microsomes from phenobarbital pretreated rabbits.

SOURCE: Uehleke et al., 1977.

pathway) and is not greatly increased with aerobic metabolism, the major pathway for metabolism of chloroform, which is O_2 dependent (Figure 4-6).

Covalent binding occurs preferentially to lipids and proteins of the endoplasmic reticulum proximate to P_{450} system for metabolism. However, considerable covalent binding from chloroform metabolites occurs in other cell fractions of liver and kidney, particularly to mitochondria (Uehleke and Werner, 1975; Hill et al., 1975). Hill et al. (1975) found when C57BL male mice were injected interperitoneally with 0.07 ml/kg ^{14}C-chloroform in oil and sacrificed 12 hours later, that in the liver, 50% of the radioactivity was irreversibly bound to microsome, 23% to mitochondria, 25% to cytosol, and <2% to nuclei; for kidney, 38% of radioactivity was bound to microsome, 39% to mitochondria, 22% to cytosol, and <2% to nuclei. A similar distribution was found in male NMRI mice by Uehleke and Werner (1975), who observed minimal binding to microsomal RNA but significant binding to nicotine-adenine nucleotides. The data of Ilett et al. (1973), shown in Figure 4-9,

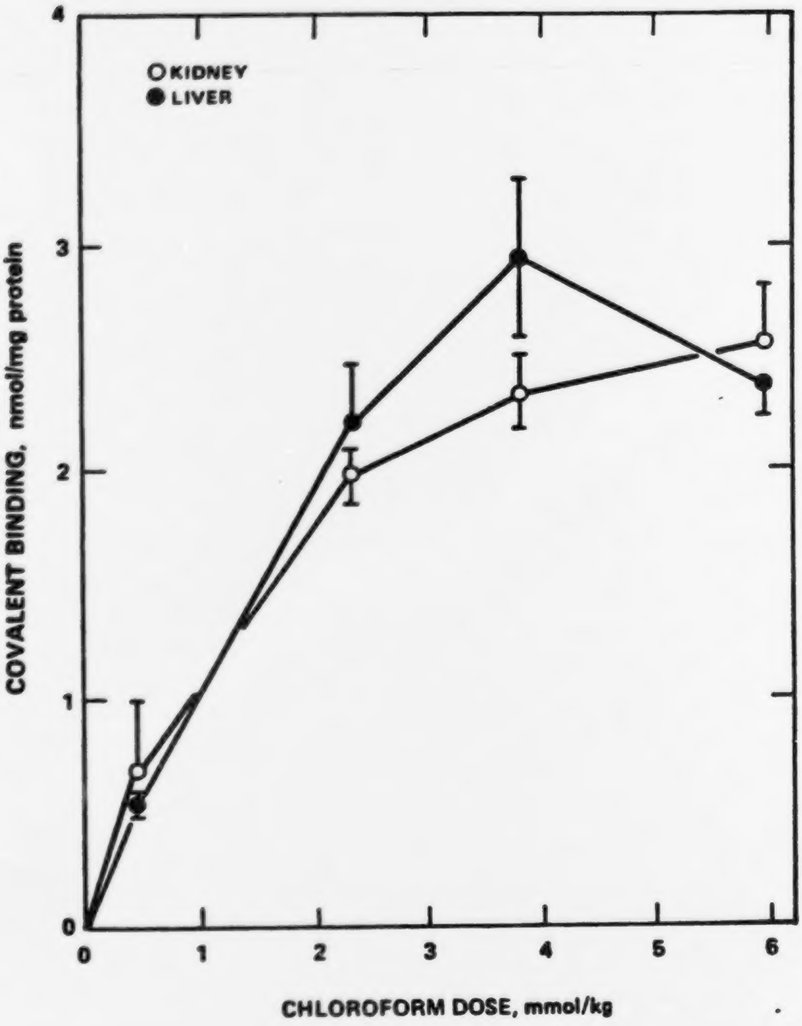

Figure 4-9. Effect of increasing dosage of i.p.-injected ^{14}C-chloroform on extent of covalent binding of radioactivity *in vivo* to liver and kidney proteins of male mice 6 hours after administration.

Source: Ilett et al. (1973).

demonstrate that in C57 BL/6 mice, the amount of covalent binding in liver and kidney microsomal fractions increases proportionally with the chloroform dose.

4.6.1.1. Genetic Strain Difference--Hill et al. (1975) described in mice two genetic variations in chloroform toxicity paralleling genetic differences in covalent binding in liver and kidney. In one inbred strain (DBA/2), the male animals were 4 times more sensitive to the lethal effects of oral doses of chloroform (LD_{50} of 0.08 ml/kg) than the second strain (C57 BL/6, LD_{50} of 0.33 ml/kg). Males of the F_1 hybrid strain ($B6D2F_1$/J) had an intermediate LD_{50} of 0.2 ml/kg, midway between those of the two parental strains. The susceptibility of DBA mice was related to a dose-dependent necrosis of the proximal convoluted renal tubules. However, mice of all three genotypes that received >0.17 ml/kg chloroform exhibited both renal tubular necrosis and hepatic centrolobular necrosis. Males and females of the same strain exhibited similar dose thresholds to hepatic damage, but females died of chloroform-induced hepatic damage without developing renal lesions. This sex-related absolute difference is dependent on androgen profile of the mice; testosterone-treated females become sensitive to renal toxicity (Bennet and Whigham, 1964; Culliford and Hewitt, 1957; Eschenbrenner and Miller, 1945b).

Table 4-13 shows the extent of covalent binding in liver and kidney of these three strains after a single intraperitoneal injection of ^{14}C-chloroform (0.07 ml/kg) to the males. Kidney homogenates from DBA/2J male mice, more sensitive to renal necrosis, contained more than 2-fold as much radioactivity as those from resistant C57BL/6J; covalent binding in the F_1 hybrid was intermediate, as expected. A significant difference was also noted in labeling of kidney subcellular fractions. While all subcellular fractions of susceptible male DBA mice were labeled to a greater extent than

TABLE 4-13. MOUSE STRAIN DIFFERENCE IN COVALENT BINDING OF RADIOACTIVITY FROM ^{14}C-CHLOROFORM[a]

Tissue	Specific activity relative to C57BL		
	DBA	F_1	C57BL
	Tissue homogenates		
Liver	0.82	0.96	1.00
Kidney	2.41	1.64	1.00
	Subcellular fractions		
Liver			
Nuclei	0.67	0.76	1.00
Mitochondria	1.14	1.14	1.00
Microsomes	0.64	0.73	1.00
Cell sap	0.98	1.09	1.00
Kidney			
Nuclei	2.20	1.67	1.00
Mitochondria	3.67	1.97	1.00
Microsomes	1.74	1.44	1.00
Cell sap	1.65	1.23	1.00

[a] Adult male mice of each genotype given ^{14}C-chloroform (0.07 ml/kg) intraperitoneally and sacrificed 12 hr later. Genotype comparisons are given as ratio of radioactivity to C57BL = 1.

SOURCE: Hill et al., 1975.

F_1 or C57BL strains, the greatest increase was in labeling of the mitochondrial fraction.

In the liver, the distribution of covalent binding was generally opposite to that observed in the kidneys (Table 4-13), but neither liver homogenates nor subcellular fractions showed significant strain differences.

4.6.1.2. Sex Difference--Kidneys of male mice are known to covalently bind more ^{14}C-chloroform radioactivity than do those of females, but females bind more in the liver than males (Taylor et al., 1974; Ilett et al., 1973) (see Tables 4-7 and 4-14). Table 4-15 shows that pretreatment of male mice with phenobarbital increases covalent binding in the liver but not in the kidney (Ilett et al., 1973). A similar observation has been made by Kluwe et al. (1978) in male mice. They found that phenobarbital increased liver but not kidney microsomal activity; 3-methylcholanthrene, dioxin, and PCGs increased both liver and kidney microsomal enzyme activities. From the renal and hepatic toxicity profile to chloroform displayed by mice treated with these various inducers, these investigators concluded that the chloroform metabolite(s) responsible for hepatic damage is probably generated in the liver, and the metabolite(s) responsible for renal damage is generated in the kidney. However, it has now been firmly established that chloroform is metabolized by the kidney to phosgene and to other reactive compounds by enzyme pathways similar to those in the liver (Pohl et al., 1984; Branchflower et al., 1984; Smith and Hook, 1984; for a discussion, see Section 5.3.2., Nephrotoxicity).

4.6.1.3. Inter-species Difference--In addition to intra-species strain (mice) differences in covalent binding noted above, Uehleke and Werner (1975) have also observed an apparent inter-species difference. Figure 4-10 shows the *in vitro* binding of radioactivity from ^{14}C-chloroform by microsomal preparations from rat, mouse, rabbit, and man. Human and rabbit microsomes have the highest rate of covalent binding from chloroform, with the mouse followed by the rat considerably lower. Inter-species differences in the covalent binding rates for carbon tetrachloride were small. These species differences in binding of chloroform metabolites to protein and lipid

TABLE 4-14. IN VIVO COVALENT BINDING OF RADIOACTIVITY FROM $^{14}CHCl_3$ IN LIVER AND KIDNEY OF MALE AND FEMALE C57BL/6 MICE (nmoles/mg protein ± S.E.)[a]

	Male	Female
Liver	2.92 ± 0.35	3.66 ± 0.39
Kidney	2.34 ± 0.16	0.39 ± 0.02

[a] Mice were sacrificed 6 hr after intraperitoneal administration of 3.72 nmoles/kg of $^{14}CHCl_3$.

SOURCE: Ilett et al., 1973.

TABLE 4-15. IN VITRO COVALENT BINDING OF RADIOACTIVITY FROM $^{14}CHCl_3$ TO MICROSOMAL PROTEIN FROM LIVER AND KIDNEY OF MALE AND FEMALE C57BL/6 MICE (pmoles/mg protein/5 min ± S.E.M.)

	Pretreatment	Liver	Kidney
Male	NA	572 ± 54	44.6 ± 4.1
Male	Phenobarbital	1454 ± 143	41.0 ± 3.2
Female	NA	419 ± 20	14.6 ± 2.5

NA = not applicable.

SOURCE: Ilett et al. 1973.

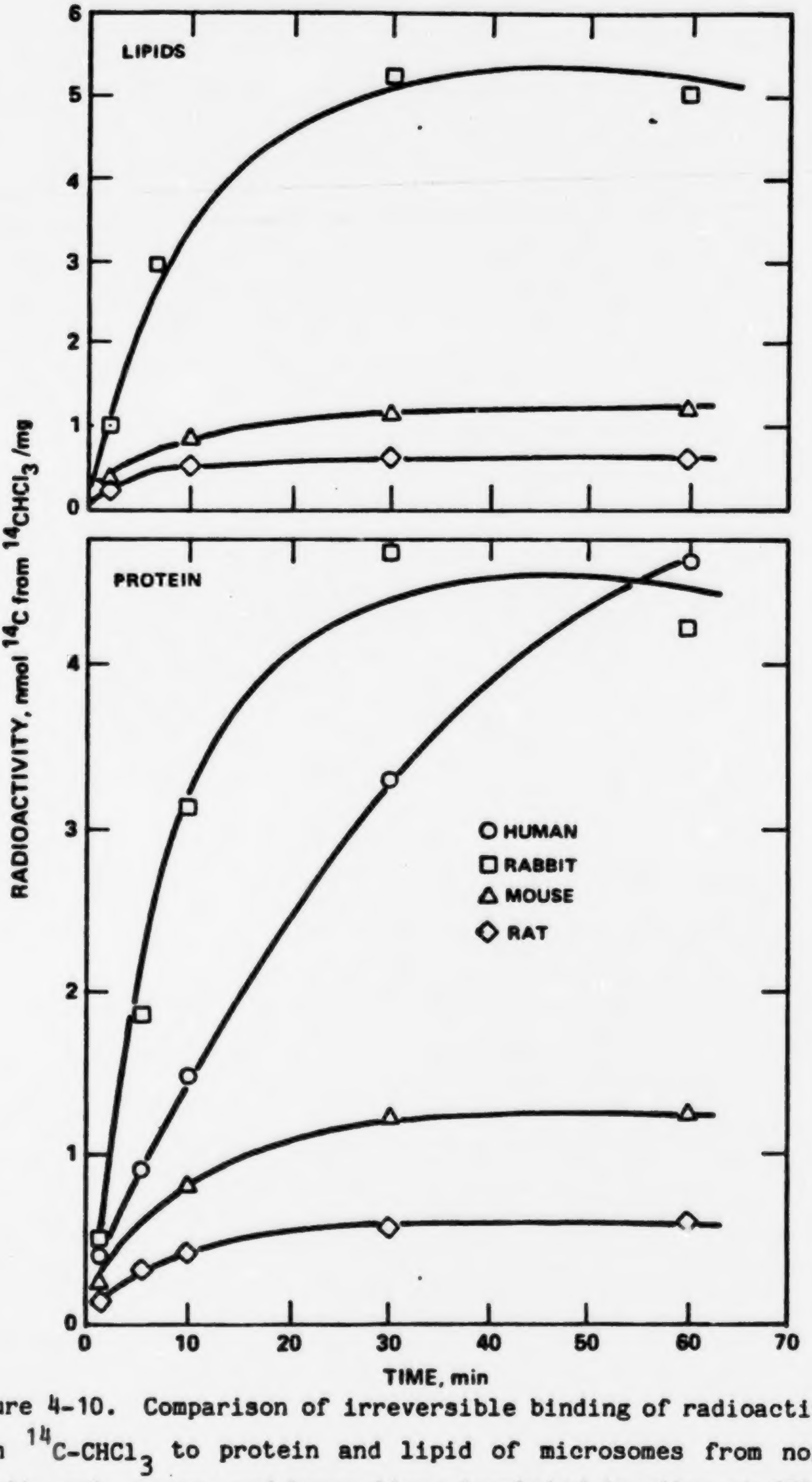

Figure 4-10. Comparison of irreversible binding of radioactivity from $^{14}C-CHCl_3$ to protein and lipid of microsomes from normal rabbit, rat, mouse, and human liver incubated *in vitro* at 37°C in O_2.

Source: Uehleke and Werner (1975).

in vitro do not, however, parallel the species differences in metabolism of chloroform in vivo, as measured by the conversion of chloroform to CO_2 (Table 4-9); in vivo, the mouse has the greatest capacity to metabolize chloroform (80%), followed by rats (65%), nonhuman primates (20%), and man (30 to 50%).

4.6.1.4. Age Difference--Uehleke and Werner (1975) have shown that irreversible protein binding of radioactivity from ^{14}C-chloroform and ^{14}C-carbon tetrachloride to liver microsomes of newborn rats (18 hours old) is low compared to that of microsomes from adult rats (32 days); however, the binding was shown to be proportional to P_{450} content of the microsomes, which was proportionally low in microsomes from newborn rats.

4.6.2. Nucleic Acids

P_{450} systems activate chloroform and carbon tetrachloride in vivo and in vitro to reactive metabolites that extensively covalently bind to proteins and lipids, but do so only minimally to nucleic acid (Uehleke et al., 1977; Wolf et al., 1977; Uehleke and Werner, 1975; Fowler, 1969; Reynolds, 1967), unlike many other carcinogens that bind DNA. Reitz et al. (1980) measured DNA alkylation in liver and kidneys of mice after an oral dose of 240 mg/kg ^{14}C-chloroform (specific activity not given) and found values of 3×10^{-4} and 1×10^{-4} mol % for liver and kidney DNA, respectively. These workers judged that chloroform has very little direct interaction with DNA when compared to known carcinogens, as reported in the literature for dimethylnitrosamine (3.5×10^{-1} mol % alkylation, liver DNA), dimethylhydralazine (2.6×10^{-2}, colon DNA) and N-methyl-N-nitrosourea (1.5×10^{-1}, brain DNA) but given by parenteral routes (Pegg and Hui, 1978; Cooper et al., 1978; Kleihues and Margison, 1974). The failure of chloroform or carbon tetrachloride reactive species to significantly bind DNA has been ascribed to their short half-life compared to epoxides and to their lack of nuclear penetration. Recently, however, Diaz Gomez and Castro (1980) have shown that highly purified rat liver nuclear preparations are able to anaerobically activate carbon tetrachloride, and to aerobically activate chloroform to reactive metabolites that bind to nuclear lipids and proteins. Their data, given in Table 4-16, show that activity in nuclear preparations is smaller than in microsomes, but within the same order of magnitude. These results might be relevant to the hepatocarcinogenic effects of chloroform and carbon tetrachloride in mice and rats, since the nuclear targets (DNA, RNA, nuclear proteins) are in the immediate neighborhood sites of activation, thus making unnecessary the present assumption that the highly reactive intermediates ($\cdot CCl_3$, phosgene, malonaldehyde, or carbene), produced at the endoplasmic reticulum, must travel to the nucleus.

Pereira et al. (1984) have investigated the effects of chloroform on hepatic and renal DNA synthesis in rats and mice and on ornithine decarboxylase (OD) activity, which is a marker enzyme for cellular proliferation, DNA synthesis, and tumorigenesis. Chloroform given i.p. induced a dose-dependent increase of hepatic OD activity 18 hr after injection. For mice a 10-fold increase of OD activity was found at a maximal dose of 375 mg/kg chloroform, and in rats a 52-fold increase was found at 750 mg/kg. Hepatic and renal DNA synthesis rate (as measured by ^3H-thymidine incorporation) was increased in mice but decreased in rats. Thus, OD activity increase in rat liver was not paralleled by an increase in DNA synthesis. Furthermore, minimal microscopic changes were detected in the liver and kidney under the conditions of the experiments. These investigators interpreted their results to mean that the induction by chloroform of liver and renal OD activity was not associated with a cellular

TABLE 4-16. COVALENT BINDING OF RADIOACTIVITY FROM ^{14}C-CHLOROFORM AND ^{14}C-CARBON TETRACHLORIDE IN RAT LIVER NUCLEAR AND MICROSOMAL INCUBATION *IN VITRO* (pmol/mg ± S.D.)

	Incubation condition	Protein	Lipid
^{14}C-CCl_4	N_2		
Nuclear		21.9 ± 2.5	147 ± 12
Microsomal		50.3 ± 4	190 ± 11
^{14}C-$CHCl_3$	O_2		
Nuclear		27.0 ± 3	20 ± 3
Microsomal		68.0 ± 9	57 ± 8

SOURCE: Diaz Gomez and Castro, 1980.

regenerative response and hence their results do not support a non-genotoxic mechanism for chloroform tumorigenesis.

4.6.3. Role of Phosgene

Phosgene is a prominent intermediate of both chloroform and carbon tetrachloride metabolisms (Figures 4-6, 4-7). It is known to be highly reactive and toxic to cells and tissues (Pawlowsky and Frosolono, 1977), and its two highly reactive chlorines suggest that it could act on cellular macromolecules similar to bifunctional alkylating agents. Reynolds (1967) showed that ^{14}C-phosgene, given to intact rats, labeled liver protein (and lipids to a smaller extent). The pattern of labeling was quite different from that of ^{14}C-carbon tetrachloride and more similar to ^{14}C-chloroform. Moreover, ^{36}Cl-carbon tetrachloride radioactivity was also stably incorporated into liver lipid and protein, pointing to the $\cdot CCl_3$ radical rather than phosgene as the reactive form for carbon tetrachloride that labels lipid. Cessi et al. (1966) also reported that ^{14}C-phosgene labeled terminal amino group of polypeptides in a manner similar to in vivo protein labeling produced by carbon tetrachloride.

4.6.4. Role of Glutathione

Ekstrom and Hogberg (1980) found that chloroform, in freshly isolated rat liver cells, induced depletion of cellular glutathione. Brown et al. (1974) demonstrated that exposure of rats to an atmosphere of 0.5% chloroform for 2 hours markedly decreased glutathione (GSH) in the liver when the animals were pretreated with phenobarbital to stimulate metabolism. GSH liver content of untreated rats was not decreased. Phenobarbital pretreatment has been shown to markedly potentiate toxicity of both chloroform and carbon tetrachloride in rats (Docks and Krishna, 1976; Cornish et al., 1973; McLean, 1970; Scholler, 1970). However, it has not been possible to detect a decrease in the liver glutathione levels following administration of carbon tetrachloride or trichlorobromomethane (Docks and Krishna, 1976; Boyland and Chasseaud, 1970). Sipes et al. (1977) have shown that the addition of GSH liver microsomes from phenobarbital pretreated rats incubated in vitro with ^{14}C-labeled halocarbons, chloroform, carbon tetrachloride, and trichloromomethane, inhibited covalent binding $\simeq$80% for all three compounds. Their results, given in Table 4-17, also show the effects of anaerobic and aerobic conditions on covalent binding. The reduction in binding of chloroform by an atmosphere of N_2 suggests that its bioactivation is mediated by a cytochrome P_{450} oxidative pathway to phosgene (Figure 4-6), while the enhanced binding of carbon tetrachloride in N_2 reflects P_{450} mediated reductive pathways (Figure 4-7) and formation of free radical. These investigators suggest that in phenobarbital-treated animals, chloroform depletes liver GSH by the formation of conjugate between the

TABLE 4-17. EFFECT OF GLUTATHIONE, AIR, N_2 OR $CO:O_2$ ATMOSPHERE ON THE IN VITRO COVALENT BINDING OF CCl_4, $CHCl_3$ and $CBrCl_3$ TO RAT LIVER MICROSOMAL PROTEIN (pmoles ^{14}C-bound/mg microsomal protein/minute)

	Substrate[a]		
Incubation conditions	CCl_4	$CHCl_3$	$CBrCl_3$
Air	97 ± 10	59 ± 5	1456 ± 66
N_2	310 ± 51	21 ± 1	1370 ± 143
$CO:O_2$ (8:2)	18 ± 1	20 ± 1	853 ± 62
SKF 525 A (0.5 mM), Air	109 ± 5	7 ± 7	2105 ± 159
Glutathione, Air	17 ± 2	15 ± 2	218 ± 25
NADPH omitted, Air	6 ± 1	3 ± 0	65 ± 13

[a] ^{14}C-labeled substrate is a final concentration of 1×10^{-3} M incubated at 37°C. Microsomes were from phenobarbital pretreated rats.

SOURCE: Sipes et al., 1977.

reactive intermediate phosgene and GSH (Docks and Krishna, 1976; Brown et al., 1974). In the case of carbon tetrachloride, they suggest that GSH addition in vitro (Table 4-16) also conjugates with the phosgene metabolite of carbon tetrachloride produced when incubated in air, but, in addition, GSH decreases the levels of $\cdot CCl_3$ by reducing the free radical to chloroform. In vivo, it is postulated that the oxidized glutathione may be reduced back to reduced GSH by glutatione reductase; this would explain the absence of falling liver GSH content with carbon tetrachloride in vivo (Gillette, 1972). Thus, the toxic effects of chloroform and carbon tetrachloride may be mediated through different mechanisms of covalent binding, and GSH may play different roles for these chlorocarbons in preventing covalent binding of reactive intermediates of metabolism.

Chloroform and carbon tetrachloride are known to cause greater liver damage in fasted animals than in fed animals (Diaz Gomez et al., 1975; Jaeger et al., 1975; Krishnan and Stenger, 1966; Goldschmidt et al., 1939; Davis and Whipple, 1919). For chloroform, a decreased content of hepatic GSH from fasting has been postulated to be responsible for the increased susceptibility of fasted mice (Docks and Krishna, 1976; Brown et al., 1974). Nakajima and Sato (1979) have recently offered an additional explanation. These investigators studied the metabolism of the chlorocarbons *in vitro* with microsomes from livers of fasted rats, and found that the disappearance of chloroform from incubation increased 3-fold for a 24 hour fast, although fasting produced no significant increase in the microsomal protein and cytochrome P_{450} liver contents (Table 4-18). Similar results were obtained for carbon tetrachloride. These observations suggest that the increased toxicity of chloroform and carbon tetrachloride from food deprivation may be due not only to decreased GSH, but also to a greater production of reactive intermediates and covalent binding to cellular macromolecules.

4.7 SUMMARY

At ambient temperatures, chloroform is a volatile liquid with high lipid solubility and appreciable solubility in water. Hence, chloroform is readily absorbed into the body through the lungs and intestinal mucosa; the portals of entry with exposure from air, water and food. Few data are available on the pharmacokinetics of absorption and excretion of chloroform in man, particularly at the low exposure concentrations expected in ambient air and drinking water. However, studies show absorption from the gastrointestinal tract in man, monkeys, rats and mice is rapid and complete, occurring by

TABLE 4-18. EFFECTS OF 24-HOUR FOOD DEPRIVATION ON CHLOROFORM AND CARBON TETRACHLORIDE *IN VITRO* MICROSOMAL METABOLISM, PROTEIN, AND P-450 LIVER CONTENTS OF RATS

	Male			Female		
	Fed	Fasted	Ratio	Fed	Fasted	Ratio
	Metabolism, nmole/g/min					
Chloroform	19.7 ± 2.6	55.1 ± 7.5	2.8	15.3 ± 6.8	39.3 ± 2.5	2.6
Carbon tetrachloride	1.9 ± 0.2	5.9 ± 0.8	3.1	1.1 ± 0.5	4.5 ± 0.3	4.1
	Protein content, mg/kg liver					
	27.7 ± 3.7	23.0 ± 2.7	NR	22.5 ± 1.5	23.7 ± 1.7	NR
	P-450, nmol/mg protein					
	0.842 ± 0.123	0.823 ± 0.03	NR	0.638 ± 0.051	0.673 ± 0.044	NR

NR = not reported.

SOURCE: Nakajima and Sato, 1979.

first-order passive absorptive processes. A dose-dependent first-pass effect with pulmonary elimination of unchanged chloroform occurs with oral ingestion in man, thus decreasing the amount of chloroform reaching the systemic circulation. In rats, the kinetics of peroral absorption are also influenced by the dosing vehicle; the absorption rate is decreased for chloroform given in corn oil vehicle as compared to an aqueous solution. Pulmonary uptake and elimination occur also by first-order diffusion processes with three distinct components with rate constants corresponding to tissue loading or desaturation of at least three major body compartments. Half-times in man have been found to be approximately 14-30 minutes, 90 minutes, and 24-36 hours, respectively. The longest half-time is associated with the lipids and the adipose tissue compartment. During inhalation exposure, at equilibrium with inspired air concentration, the blood/air partition coefficient is about 8 at 37°C and the adipose tissue/blood partition coefficient is 280 at 37°C. The quantity of chloroform absorbed is dependent also on body weight and fat content of the body.

Tissue distribution of chloroform is consistent with its lipophilic nature and modest water solubility. This chloroalkane readily crosses the blood brain and placental barriers and distributes into breast milk. Concentrations occurring in all major tissue organs are dose related to inspired air concentrations or to oral dosage. Relative tissue concentrations occur in the order of adipose tissue > brain > liver > kidney > blood.

Elimination of chloroform from the body occurs by two major and parallel occurring processes: (1) pulmonary elimination of unchanged chloroform by first order kinetics and (2) metabolism of chloroform. Chloroform is metabolized in the liver and to a lesser extent in the kidneys and other tissues. Metabolism is dose-dependent and saturable, with a greater proportion of small doses being metabolized. There are striking differences in the pharmacokinetics and quantitative metabolism of chloroform in man as compared to other animals. For large steady-state body burdens, 30-40% is metabolized by man, 20% by the nonhuman primate, >65% by the rat, and >85% by the mouse. Metabolism produces phosgene and other putative reactive metabolites that covalently bind extensively to cellular lipids and proteins, although not significantly to DNA or other nucleic acids. The intensity of metabolite binding and organ localization parallel the acute cellular toxicity of chloroform in liver and kidney observed in experimental animals. Both binding and toxicity are highly dependent on animal species and genetic strain, as well as on sex and age. An additional variable is the tissue level of reduced glutathione, which plays an important role in protecting against both binding and toxicity. Conversely, inducers of hepatic and renal P_{450} metabolizing systems increase binding and toxicity.

4.8 REFERENCES FOR CHAPTER 4

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5. TOXICITY

5.1. EFFECTS OF ACUTE EXPOSURE TO CHLOROFORM

In both humans and experimental animals, characteristic effects of acute exposure to chloroform are depression of the central nervous system and hepatic damage. Renal and cardiac effects also occur. The systemic toxic effects of chloroform appear to be similar regardless of whether exposure or administration occurred by inhalation, oral, or parenteral routes. The only systemic effect documented for dermal administration, however, is renal damage.

5.1.1. Humans

5.1.1.1. Acute Inhalation Exposure in Humans--Information on the effects of acute inhalation exposure of chloroform on humans has been obtained primarily during its use as an inhalation anesthetic. The relationship of the concentration of chloroform in inspired air and blood to anesthesia is described in Table 5-1 (Goodman and Gilman, 1980). Concentrations of chloroform used for the induction of anesthesia were in the range of 2-3 volumes % (20,000-40,000 ppm), followed by lower maintenance levels (NIOSH, 1974; Adriani, 1970).

Chloroform inhalation has a depressive effect on the central nervous system. Excitement due to release of inhibitions is followed by progressive depression of the cortex, higher centers, medulla and spinal cord (Wood-Smith and Stewart, 1964). Centers controlling temperature regulation, respiration, vomiting, vasomotor, and vagal activity are all depressed (Adriani, 1970).

The cardiovascular system is also affected by anesthetic use of chloroform. The myocardium is directly depressed in deeper planes of anesthesia. A blood level sufficient to cause respiratory failure may also

TABLE 5-1. RELATIONSHIP OF CHLOROFORM CONCENTRATION IN INSPIRED AIR AND BLOOD TO ANESTHESIA

	In inhaled air, volumes %	In blood, mg%
Not sufficient for anesthesia	<0.15	<2
Light anesthesia (after induction)	0.15 to 0.20	2 to 10
Deep anesthesia	0.20 to 1.50	10 to 20
Respiratory failure	2.0	20 to 25

*SOURCE: Goodman and Gilman, 1980.

cause cardiac arrest. In addition, chloroform sensitizes the autonomic tissues of the heart to epinephrine, causing arrhythmias. It has been found that under chloroform anesthesia regarded as normal, the heart is subject to arrhythmias and extrasystoles (Kurtz et al., 1936; Orth et al., 1951). Orth et al. (1951) found a high incidence of ventricular arrhythmias, 20 of 52 cases investigated, and four cases of temporary cardiac arrest. Blood pressure is lowered by chloroform as a result of a 3-fold action: cardiac slowing due to vagal stimulation, depression of the vasomotor center, and dilation of splanchnic blood vessels (Krantz and Carr, 1965).

Respiratory effects of chloroform inhalation include increased rate and depth of respiration during induction and in light anesthesia, and decreased minute volume exchange in deeper planes of anesthesia. The Hering-Breuer reflex remains active. Bronchial smooth muscle is relaxed and secretions are increased. Laryngeal spasms are caused by high concentrations (Adriani, 1970).

In the gastrointestinal tract, chloroform markedly stimulates the flow of saliva during induction and recovery, but salivation is inhibited in deeper planes of anesthesia (Goodman and Gilman, 1980). The pharyngeal or gag reflex is depressed. Under anoxic conditions, pharyngeal muscle spasms result in stertorous respiration and thick mucus is excreted (Adriani, 1970). Stomach movements are decreased or abolished as tone is reduced. Gastric secretory activity is inhibited or abolished. Post-anesthetic dilation of the stomach occurs in nearly all cases. Nausea and vomiting often occur during recovery from anesthesia. The mechanism is central rather than local, but may be due in part to irritation of the stomach by swallowed vapor (Goodman and Gilman, 1980). Intestinal tone, motility, and secretory activity are inhibited or abolished (Adriani, 1970).

In the urinary tract, chloroform anesthesia results in a decrease in urine flow, possibly due to the release of antidiuretic hormone and renal vasoconstriction, leading to a decrease in renal blood flow and glomerular filtration. Polyuria occurs after recovery (Goodman and Gilman, 1980). Chloroform anesthesia may be followed by albuminuria and glycosuria. Post-operative urine retention occurs frequently. Renal tubular necrosis has been found in cases of severe poisoning (Wood-Smith and Stewart, 1964).

During obstetric use of chloroform, uterine contractions are only slightly decreased by light anesthesia, but are markedly inhibited in deeper planes. Chloroform rapidly crosses the placental barrier, and respiratory depression in the infant is likely to occur (Wood-Smith and Stewart, 1964).

Chloroform anesthesia also has metabolic effects in humans. A rise in blood glucose accompanies anesthesia. Levels may rise ≥2-fold and remain elevated for several hours. The liver glycogen falls coincident with the rise in blood sugar. This, in turn, is a result of the release of epinephrine from the adrenal medulla during the period of excitation. There is also a decrease in glucose utilization in the periphery (Goodman and

Gilman, 1980; Krantz and Carr, 1965). Acidosis occurs, characterized by a fall in plasma biocarbonate and phosphate.

Chloroform is acutely toxic to the liver, although in so-called delayed chloroform poisoning, the full effects of damage done during and shortly after administration are not seen for 24-48 hours. The glycogen content of the liver is rapidly depleted; three-fourths in the first half-hour and less rapidly thereafter. There is centrilobular and, in severe cases, mid-zonal and massive necrosis. Cells which survive show fatty degeneration. Symptoms include progressive weakness, prolonged vomiting, delirium, coma, and death. They develop from the first to the third day after exposure. Jaundice, increased serum bilirubin, bile in the urine, reduction in liver function, increased nitrogen excretion, lowered blood prothrombin and fibrinogen, and the appearance of leucine, tryosine, acetone, and diacetic acid in the urine are some of the more prominent findings. The hemorrhagic tendency is due to reduced prothrombin formation by the injured liver. Death usually occurs on the fourth or fifth day, and autopsy reveals degeneration and necrosis of liver tissue, most marked around the central veins (Goodman and Gilman, 1980; Wood-Smith and Stewart, 1964).

Hematologic effects due to acute chloroform inhalation are seen during anesthesia. Erythrocytes are increased in number as the spleen is constricted and red blood cells are extruded into the circulation. Leukocytes are increased in number during the post-anesthetic period, reaching a maximum within 24 hours and returning to normal in 48 hours. There is an increase in polymorphonuclear cells. Platelets remain unchanged. One half-hour after exposure, there is a decrease in clotting time. Prothrombin time is increased. Prothrombin synthesis is impaired by liver toxicity as previously noted (Adriani, 1970).

The effects of chloroform on the eye include dilation of the pupils, with reduced reaction to light as well as reduced intraocular pressure (Sax, 1979; Winslow and Gerstner, 1978).

Signs of chloroform poisoning include a characteristic sweetish odor on the breath, cold and clammy skin, and dilated pupils (Winslow and Gerstner, 1978). Nausea and vomiting commonly occur. Ketosis, due to incomplete oxidation of fats, as well as a rise in blood sugar, accompanies chloroform intoxication. Initial excitation alternating with apathy is followed by prostration, unconsciousness, and possible death due to cardiac and central nervous system depression (Winslow and Gerstner, 1978).

The above discussion presents observations made on the effects of chloroform inhalation during general anesthesia. Information on the effects of experimental acute inhalation exposure of chloroform in humans is limited to the work of Lehman and Hasegawa (1910) and Lehman and Schmidt-Kehl (1936) as reviewed by NIOSH (1974). The duration of exposure was ≤30 minutes and only the subjective responses of the subjects were measured. The dose-response relationships as tabulated by NIOSH (1974) are presented in Table 5-2.

5.1.1.2. Acute Oral Exposure in Humans--Case reports of suicides (Piersol et al., 1933; Schroeder, 1965) and of recreational abuse (Storms, 1973) of chloroform present some information on the effects of acute imbibition. A fatal dose of ingested chloroform may be as little as one-third of an ounce (10 ml) (Schroeder, 1965). The initial effect is usually unconsciousness and possibly death (within 12 hours without treatment) due to respiratory or cardiac arrest. If the patient survives, delayed effects are observed within 48 hours after regained consciousness. These symptoms include vomiting, anorexia, jaundice, liver enlargement, albinuria, ketosis, ketonuria and

TABLE 5-2. DOSE-RESPONSE RELATIONSHIPS

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160 ppm (0.8 mg/L) for unspecified time - no odor

205 ppm (1.0 mg/L) for unspecified time - light transient odor

390 ppm (1.9 mg/L) for 30 minutes -light transient odor

920 ppm (4.5 mg/L) for 7 minutes - stronger, lasting odor; dizziness, vertigo after 3 minutes

680 ppm (3.3 mg/L) to 1000 ppm (5.0 mg/L) for 30 minutes - moderately strong odor; taste

1100 ppm (5.4 mg/L) for 5 minutes - still stronger, permanent odor; dizziness, vertigo after 2 minutes

1400 ppm (6.6 mg/L) to 1800 ppm (8.57 mg/L) for 30 minutes - stronger odor, tiredness, salivation, giddiness, vertigo, headache, taste

3000 ppm (14.46 mg/L) for 30 minutes - all above plus pounding heart, gagging

4300 ppm (20.8 mg/L) to 5000 ppm (25 mg/L) for 20 minutes - dizziness and light intoxication

5100 ppm (25 mg/L) for 20 minutes - dizziness and light intoxication

7200 ppm (35.3 mg/L) for 15 minutes - dizziness and light intoxication as above but more pronounced

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SOURCE: Lehman and Hasegawa (1910) and Lehman and Schmidt-Kehl (1936).

glucosuria, hemorrhage due to lowered blood fibrinogen and prothrombin, reduced serum bicarbonate, increased blood sugar, coma and possible death. Upon autopsy, extensive hepatic centrilobular necrosis is evident.

5.1.1.3. Acute Dermal and Ocular Exposure in Humans--Chloroform is absorbed through the intact skin (von Oettingen, 1964). Application of chloroform to the skin is followed after 3 minutes by a pungent and burning pain reaching its maximum after 5 minutes, associated with erythema, hyperemia, and finally vesication (Oettel, 1936). Exposure of the eye to concentrated chloroform vapors causes a stinging sensation. Splashing the substance into the eyes evokes burning, pain, and redness of the conjunctival tissue. The corneal epithelium is sometimes impaired; however, regeneration starts rapidly and leads to full recovery within 1-3 days (Winslow and Gerstner, 1978).

5.1.2 Experimental Animals

5.1.2.1. Acute Inhalation Exposure in Animals--Tolerance of animals to chloroform has been summarized by Lehmann and Flury (1943) and by Sax (1979). Similar central nervous system effects are seen in animals at approximately the same magnitude of exposure that produced these effects in humans. In mice, exposure to 2500 ppm for 2 hours produced no obvious effects, 3100 ppm for 1 hour produced slight narcosis, while 4000 ppm induced deep narcosis within one-half hour. Only slight symptoms are seen at 2000-6000 ppm for longer exposures. Fatal exposures were 4100-8200 ppm for mice, 12,300 ppm for rabbits, and 16,300-20,500 ppm for guinea pigs (duration of fatal exposures not specified). In cats, exposure to 7200 ppm resulted in disturbance of the equilibrium after 5 minutes, light narcosis after 60 minutes, and deep narcosis after 93 minutes of exposure. Exposure to 21,500 ppm produced disturbances in equilibrium after 5 minutes, light narcosis after 10 minutes, and deep narcosis in cats after 13 minutes of exposure.

Kylin et al. (1963) described the effects of a single exposure of mice to 100, 200, 400, or 800 ppm of chloroform for 4 hours. The mice exposed to 100 ppm did not develop demonstrable liver necroses, although moderate fatty infiltration of the liver was noted. In mice exposed to 200 ppm, some necrotic areas appeared in the liver and there was an increase in serum ornithine-carbamyl transferase. Exposure to chloroform at 400 and 800 ppm resulted in increased hepatic necrosis and serum enzyme activity.

More recent data regarding toxic effects of acute inhalation exposure to chloroform were presented by Wood et al. (1982), although the study was designed primarily to investigate the role of hydrogen bonding in the anesthetic mechanism. Groups of mice in a rotating cage were given a single exposure of up to 3 hours of varying concentrations of chloroform or deuterated chloroform, each concentration being held constant for about 20 minutes and then being raised until the mice had lost their righting reflex. The concentration was then lowered to 1/2 the ED_{50} ($\approx$1500 ppm) where it remained until the mice had regained their righting reflex. The duration of these manipulated exposures never exceeded 3 hours. Only 4 of 47 mice given chloroform gained the righting reflex; indeed, some mice died or were comatose. Upon histological examination of animals sacrificed 3-6 hours after exposure, mild hepatic centrilobular necrosis and very mild renal tubular necrosis was observed. The animals receiving deuterated chloroform survived for 24 hours, after which they were sacrificed. The liver and kidney lesions in these mice were more severe, perhaps owing to the longer survival time, which may have allowed these lesions to develop.

5.1.2.2. Acute Oral Exposure in Animals--Kimura et al. (1971) performed acute oral toxicity studies in newborn (5-8 g), 14-day-old (16-50 g), young adult (80-160 g), and older adult (360-470 g) rats. The chloroform was given in undiluted form to unfasted rats. LD_{50} values in ml/kg (95% confidence limits) were reported as follows: 14-day-old, 0.3 (0.2-0.5); young adult, 0.9 (0.8-1.1); and older adult, 0.8 (0.7-0.9). The young and older adult rats were males; the other two groups contained rats of both sexes. When compared with 15 other solvents included in this study, the LD_{50} values for chloroform were the lowest in the two adult groups and next to lowest in the 14-day-old rats. Only a rough approximation of the LD_{50} could be obtained for the newborn rats; volumes of 1 ml/kg body weight were generally fatal. Lower volumes could not be measured with any degree of accuracy and were not attempted.

Torkelson et al. (1976) reported an oral LD_{50} of 2.0 g/kg (1.05-3.80) in male rats. Animals receiving as little as 0.25 g/kg showed adverse effects. Other recent studies of acute oral toxicity have reported LD_{50} values (with 95% confidence limits) of 1120 mg/kg (789-1590) in ICR male mice and 1400 mg/kg (1120-1680) in females (Bowman et al., 1978), and 908 mg/kg (750-1082) and 1117 mg/kg (843-1514), respectively, in male and female rats (Chu et al., 1980).

In a study that compared the toxicities of halogenated hydrocarbons, a single oral dose of 60 mg/kg chloroform to mice had no toxic effect (Hjelle et al., 1982).

Hill (1978) performed experiments in mice designed to study variability in susceptibility to chloroform toxicity from single oral doses based upon genetic sex differences. For three strains of mice, the LD_{50} values (ml/kg) were as follows: DBA/2J, 0.08; B6D2F1/J, 0.20; and C57BL/6J, 0.33. The animals more sensitive to chloroform-induced death were also found to be more susceptible to renal toxicity. Males were found to be more sensitive to renal damage and death than were females. This difference was related to testosterone and it was further noted that C57BL/10J males are relatively testosterone deficient in comparison to DBA males. The C57BL/6J strain used in the Hill (1978) study is closely related to the C57BL/10J strain and may, therefore, also be testosterone deficient.

In male B6C3F1 mice, severe diffuse renal necrosis occurred after a single oral dose of 240 mg/kg and focal tubular regeneration occurred after a single dose of 60 or 240 mg/kg. These effects were not seen after 15 mg/kg

(Reitz et al., 1980). Liver damage (hepatocellular necrosis and swelling with inflammatory cell infiltration) occurred only at the highest doses.

Chu et al. (1982a) studied the effects of acute oral exposure of chloroform on Sprague-Dawley rats. Groups consisted of 10 male and 10 female animals given a single oral dose of 0, 546, 765, 1071, 1500, or 2100 mg/kg of chloroform in a volume of 5 ml/kg corn oil. Clinical signs of toxicity included depression and coma, but the authors did not specify whether these signs occurred at all dose levels. Treated rats surviving for 14 days consumed less food and had depressed growth rates. Gross examination revealed increased liver and kidney weights at 1071 mg/kg. Upon comprehensive histological examination, only mild to moderate lesions, even at high doses, were observed in these organs. No changes were noted in other organs, including brain and heart. The hepatic and renal lesions were characterized by hepatocyte variations and occasional vesiculation of biliary epithelial nuclei in the liver, and by bilateral focal interstitial nephritis and fibrosis in the kidney. Changes in hematological and biochemical parameters were also observed in the 1071 and 1500 mg/kg treated groups. Cholesterol levels increased while lactate dehydrogenase activity and liver protein levels decreased. In female rats, the activity of microsomal aniline hydroxylase was induced by chloroform exposure. The numbers of lymphocytes were reduced in both males and females, as were hemoglobin and hematocrit values. Upon longer exposures of 5, 50, or 500 ppm chloroform in drinking water for 28 days, the only toxic effect observed was a decreased number of neutrophils in the highest dose-exposed rats. Examinations were performed as in the single-dose experiment.

Yannai (1983) has reported that chloroform has profound effects on the adrenal-pituitary axis, and that these effects may be a parameter in chloroform toxicities. After administration of single oral doses of chloroform (1.5 g/kg) to rats, adrenal hypertrophy developed and persisted for 12 days. Adrenal cholesterol content decreased within 3 hr (an indication of increased corticosteroid synthesis) and was accompanied by an increase of plasma corticosterone within 15 min, remaining elevated for 4 hr. The blood clearance rate of corticosterone was not changed. Since cortisteroids play an important role in the stress response, these result suggest that toxic agents such a chloroform may activate the adrenal-pituitary axis which may in turn modify the toxic response.

5.1.2.3 Acute Dermal and Ocular Exposure in Animals--Torkelson et al. (1976) found that chloroform, when applied to the skin of rabbits, produced slight to moderate irritation and delayed healing of abraded skin. When applied to the uncovered ear of rabbits, slight hyperemia and exfoliation occurred after one to four treatments. No greater injury was noted after 10 applications. One to two 24-hour applications, on a cotton pad bandaged on the shaven belly of the same rabbits, produced a slight hyperemia with moderate necrosis and a resulting eschar formation. Healing appeared to be delayed on the site as well as on abraded areas which were also covered for 24 hours with a cotton pad soaked in chloroform.

Single application of either 1.0, 2.0, or 3.98 g/kg for 24 hours under an impermeable plastic cuff held tightly around the clipped bellies of each of two rabbits did not result in any deaths. However, extensive necrosis of the skin and considerable weight loss occurred at all levels. All animals were sacrificed for study 2 weeks after exposure. All treated rabbits exhibited degenerative changes in the kidney tubules graded in intensity with dosage levels. The livers were not grossly affected.

In the same study (Torkelson et al., 1976), liquid chloroform, dropped into the eyes of three rabbits, caused slight irritation of the conjunctiva which was barely detectable 1 week after treatment. In addition, slight but definite corneal injury occurred, as evidenced by staining with fluorescein. A purulent exudate occurred for >2 days after treatment. Although started 30 seconds after instilling the chloroform, thorough washing of one eye of each rabbit with a stream of running water for 2 minutes did not significantly alter the response in the washed eyes from that of the unwashed eyes.

5.1.2.4 . Intraperitoneal and Subcutaneous Administration in Animals--The toxicity of chloroform in mice after subcutaneous administration (Kutob and Plaa, 1962b) and intraperitoneal administration (Klaassen and Plaa, 1966) has been compared with that of other halogenated hydrocarbons (Pohl, 1979). In these studies, the LD_{50} values for carbon tetrachloride, chloroform, and dichloromethane were, respectively, 200, 27.5, and 76 mmol/kg after subcutaneous administration and 20, 14, and 23 mmol/kg when given intraperitoneally. When the relative hepatotoxicity of these compounds was compared, a subcutaneous dose of 0.5 mmol/kg of carbon tetrachloride produced approximately the same degree of liver damage as 6.2 mmol/kg of chloroform. After intraperitoneal administration, these values dropped to 0.01 mmol/kg for carbon tetrachloride and 2.3 mmol/kg for chloroform. Dichloromethane did not cause significant histological changes in the liver by either route of administration. At doses that produced liver toxicity, chloroform caused kidney lesions which ranged from the presence of hyaline droplets, nuclear pycnosis, hydropic degeneration, and increased eosinophilia, to necrosis with karyolysis and loss of epithelium of the convoluted tubules.

Ilett et al. (1973) found that intraperitoneal administration of chloroform caused centrilobular hepatic necrosis in mice of both sexes, whereas renal necrosis was observed only in male mice.

5.2. EFFECTS OF CHRONIC EXPOSURE TO CHLOROFORM

A characteristic effect of chronic exposure to chloroform is hepatic damage; this effect has been documented primarily in studies with experimental animals. As was the case for acute exposure to chloroform, hepatic damage in chronic studies results from either inhalation or oral administration of this chemical. Effects on the kidneys and thyroids have also been observed in some experiments. This section will discuss both subchronic (≈90 days) and chronic exposure studies, because many of the subchronic studies were preliminary range-finding tests for the chronic studies.

5.2.1. Humans

5.2.1.1. Chronic Inhalation Exposure in Humans--Only two chronic inhalation studies that reported measurements of exposure concentrations, as well as effects on human health, were found. Neither study (Challen et al., 1958; Bomski et al., 1967) was performed adequately.

Challen et al. (1958) investigated complaints of workers (mainly women) in a plant manufacturing lozenges that contained chloroform as a principle ingredient. Before exhaust ventilation was installed, 9 of the 10 exposed workers had complained of symptoms of tiredness, dull-wittedness, depression, gastrointestinal distress, and frequent and scalding urination. Breathing-zone monitoring during simulation of "pre-ventilation" working conditions suggested that the employees had been exposed to ≈77-237 ppm. Discussions with management revealed that some of these workers had occasionally been observed to behave in a silly manner or to stagger about during the workday. Another group of workers (N = 10) had been exposed primarily to concentrations of 22-71 ppm. Eight of these workers complained of less severe symptoms. Apparently, both groups of workers had been exposed to occasional peak concentrations of ≈1163 ppm lasting 1.5-2 minutes. At least four workers in each group worked half-time. None of the five controls reported symptoms similar to those reported by the exposed workers. Eight of the higher exposure employees (77-237 ppm for 3-10 years, followed by ≈2 years without exposure), nine of the lower exposure employees (22-77 ppm for 10-24 months), and five unexposed employees submitted to physical examinations, including liver function tests (thymol turbidity, serum bilirubin, and urine urobilinogen tests). These examinations and tests revealed no evidence of any organic lesion, including liver damage, attributable to exposure to chloroform.

In humans, hepatic damage is the most common toxic effect of acute exposure to chloroform, as noted previously. According to Pohl (1979), only one report of liver abnormalities in humans after chronic exposure to chloroform has been found in the literature and no additional reports were found in the more recent literature. In this study (Bomski et al., 1967), 17 cases of hepatomegaly were found in a group of 68 industrial workers who were exposed to chloroform in concentrations ranging from 2-205 ppm for 1-4 years. These apparently were unspecified area, as opposed to breathing zone, concentrations. Three of the 17 workers with hepatomegaly were judged to have toxic hepatitis on the basis of elevated serum enzymes. The frequency of viral hepatitis among the 68 chloroform-exposed workers was higher (4.4% versus 0.38%) than the frequency among a group of inhabitants of the city, >18 years of age. This phenomenon also occurred in the 2 previous years. Ten cases of splenomegaly were also diagnosed among the 68 workers. There appears to be no comparison with incidences of these conditions in nonexposed workers.

Recently Phoon et al. (1985) have reported on the occurrence of toxic jaundice from chemical exposure of 31 factory workers to chronic, relatively low levels of chloroform. The clinical symptomatology was similar to viral hepatitis, without the high temperature or fever associated with that disease. These workers had originally been diagnosed as having viral hepatitis, but further investigation showed that each worker had been exposed to chloroform. Two outbreaks were described. Between 1973 and 1974, over a period of 9 months, 13 workers were afflicted in a large factory in Singapore manufacturing small household appliances for which a degreaser (chloroform) was used. The workers (mostly young females) complained of anorexia, nausea, vomiting and jaundice. The level of chloroform in the air was estimated at higher than 400 ppm, and blood samples showed blood chloroform levels of 0.10 to 0.20 mg%. A second outbreak of 11 cases occurred within a four-week period in 1980 and a further 7 between 1980 and 1981, all from the same section of a second factory making radios and clock radios. Preliminarily diagnosed as acute infectious hepatitis, they were all negative for hepatitis B surface antigen. These affected workers (again mostly young females) worked in the casing department where components were assembled, encased and packed for export, and where chloroform was used as a plastic adhesive. Air analysis showed levels of 14.4 to 50.4 ppm on different days. Blood samples revealed chloroform in some but not all workers. The duration of exposure to chloroform, before onset of jaundice, averaged 1 to 4 months.

5.2.1.2. Chronic Oral Exposure in Humans--There are relatively few reports of toxic effects following chronic ingestion of chloroform (Pohl, 1979), and more recent reports were not located in the literature. In one case, it was estimated that a male patient ingested 1.6-2.6 g of chloroform in a cough medicine daily for ≈10 years (Wallace, 1950). Blood and urine analyses, as well as liver function tests, indicated the individual suffered from hepatitis and nephrosis. Another report described three patients addicted to chlorodyne, a tincture containing chloroform and morphine (Conlon, 1963). Liver biopsy showed severe cellular damage in one of these individuals who had ingested 21 ml of chloroform daily for an undetermined period of time. All three displayed evidence of serious mental and physical deterioration, including peripheral neuropathy. It is not possible to determine if the adverse effects were due to chloroform, morphine, or ethanol.

More recently, the safety of a dentifrice containing 3.4% chloroform and a mouthwash containing 0.43% was assessed in studies lasting >1 year (DeSalva et al., 1975). The subjects using the dentifrice were exposed to ≈70 mg (0.047 ml) of chloroform each day, whereas the groups using the mouthwash were exposed to ≈178 mg (0.12 ml). The results of liver function tests and blood urea nitrogen determinations showed no statistical differences between control and experimental subjects.

Epidemiologic studies of humans exposed to chloroform in their drinking water have focused on carcinogenic end points, and, hence, are discussed in the chapter on carcinogenicity.

5.2.2. Experimental Animals

5.2.2.1. Chronic Inhalation Exposure in Animals--Experiments with several species of animals (Torkelson et al., 1976) give some information regarding potential effects of long-term inhalation exposure to chloroform. The animals were exposed to chloroform 5 days/week for 6 months. Exposure to 25 ppm of chloroform for up to 4 hours/day had no adverse effects in male rats as judged by organ and body weights, and by gross and histological examination of livers and kidneys. Exposure to 25 ppm for 7 hours/day, however, produced histopathological changes in the livers and kidneys of male but not female rats. These changes were characterized as lobular granular degeneration and focal necrosis throughout the liver and cloudy swelling of the kidneys. The hepatic and renal effects appeared to be reversible because rats exposed according to the same protocol, but given a 6 week recovery period following exposure, appeared normal by the criteria tested. Increasingly pronounced changes were observed in the livers and kidneys of both sexes of rats exposed to 50 or 85 ppm for 7 hours/day. Hematologic indices, clinical chemistry, and urinalysis values, tested at higher levels of exposure, were within normal limits. Each exposure group and control group had 10-12 rats/sex except for the 25 ppm, 4 hour/day group, which had 10 male rats and no females.

Similar experiments with guinea pigs (N = 8-12/sex/group) and rabbits (N = 2-3/sex/group) gave somewhat inconsistent results. Histopathological changes were observed in livers and kidneys of both species at 25 ppm but not at 50 ppm in either species, nor even at 85 ppm in guinea pigs. The results of these studies are summarized in Table 5-3.

Other reports of effects of chronic inhalation exposure to chloroform in experimental animals were not found in the more recent literature.

5.2.2.2. Chronic Oral Exposure in Animals--The data from several studies on the effects of chronic and subchronic oral exposure to chloroform are summarized in Table 5-4. Low levels of exposure (15-64 mg/kg/day, 6 days/week) have been reported to increase survival in mice and rats (Roe et al., 1979; Palmer et al., 1979), and to be associated with (1) possible transient CNS depression and mild hepatic changes in mice and rats (Jorgenson and Rushbrook, 1980; Palmer et al., 1979); (2) hepatic damage in dogs

TABLE 5-3. EFFECTS OF INHALATION EXPOSURE OF ANIMALS TO CHLOROFORM, 5 DAYS/WEEK FOR 6 MONTHS^a

		Exposure		Number in Group		
Species	Sex	ppm	hours/day	Started	Survived	Effects
rats	M	85	7	10	6	Excess mortality attributed to pneumonia on basis of gross and microscopic appearance of lungs; slight depression of final body weight; increase ($p<0.05$) in relative but not absolute weights of liver, kidneys, and testes; no effect on spleen weight; histological findings included marked centrilobular granular degeneration of the livers and cloudy swelling of the kidneys but no histopathological changes in testes; hematologic values (including differential count), urinalysis values and SGPT, SUN, and SAP values all "within normal limits"
	F	85	7	10	10	No evidence of pneumonia; final body weights and weights of liver and spleen unaffected, relative and absolute kidney weights increased; histological findings included marked centrilobular granular degeneration of the livers and cloudy swelling of the kidneys; hematologic, urinalysis, and SGPT, SUN, and SAT values all "within normal limits"
	M	50	7	10	9	Depression of final body weights ($p<0.05$); increases in relative ($p<0.05$) but not absolute weights of kidneys, spleen, and testes; histopathological changes in livers and kidneys similar to those seen at 85 ppm; hematologic values, urinalysis values, and SGPT, SUN, and SAP values all "within normal limits"
	F	50	7	10	10	Final body weights and weights of liver and spleen unaffected; increase in relative kidney weight ($p<0.05$); histopathological changes in livers and kidneys similar to those seen at 85 ppm but somewhat less marked; hematologic values, urinalysis values, and SGPT, SUN, and SAP values all "within normal limits"
	M	25	7	12	9	No effect on final body weights or weight of liver, spleen, or testes; increased relative kidney weight ($p<0.05$); lobular granular degeneration with focal areas of necrosis throughout the liver; cloudy swelling of renal tubular epithelium
	F	25	7	12	12	No statistically significant effect on body weight; increased relative but not absolute kidney and spleen weights; no histopathologic changes in kidneys and spleen; microscopic appearance of livers not specified

TABLE 5-3 (continued)

Species	Sex	Exposure ppm	 hours/day	Number in Group Started	 Survived	Effects
rats	M,F	25 plus 0 ppm for 6 weeks (recovery period)	7	12/sex	8 M, 10 F	"Normal" by the criteria tested at this dosage level (see 25 ppm above)
	M	25	1,2, or 4	10, 10, 10	7, 8, 4, respectively	No evidence of adverse effects by the criteria tested (i.e., final body weight; weights of livers, kidneys, spleen, testes; and probably gross and microscopic appearance of at least the liver and kidneys)
guinea pigs	M,F	85,50, or 25	7	8 to 12 /sex/ exposure level	50 to 92% (mortality not related to exposure)	No adverse effects at 50 or 85 ppm other than marked pneumonitis in F at 85 ppm; some histopathological changes in livers of both sexes and kidneys of M at 25 ppm (criteria tested were body weights, organ weights, and gross and microscopic appearance of organs)
rabbits	M,F	85, 50, or 25	7	2 to 3 /sex/ exposure level	0 to 1 death/group, not related to exposure	No adverse effects at 50 ppm; some histopathologic changes in kidneys and liver and pneumonitis in lungs at 25 and 85 ppm. Hematologic and clinical chemistry values within normal limits at 85 ppm (criteria tested were same as for rats)
dogs	M,F	25	7	1/sex	1/sex	No adverse effects in M; marked cloudy swelling of renal tubular epithelium and increase in capsular space in glomeruli of kidneys in F (criteria tested were same as for rats and included clinical chemistry and hematological studies)

[a] Controls for each species and sex included at least one unexposed and one air-exposed group, each comparable in number of animals to the exposed groups. In statistical comparisons of organ and body weights, values for the control group (unexposed or air-exposed) closer in body weight to the test group were used. Mortality in control groups was similar to mortality in treated groups, with the exception of excess mortality in male rats exposed to 85 ppm for 7 hours/day or to 25 ppm for 4 hours/day. No explanation was given by Torkelson et al. (1976) for the high mortality in the 4 hours/day group. Strains of animals and age or weight at the start of the experiment were not specified. Purity of the chloroform used was 99.3% (0.4% ethyl alcohol and <0.3% of an unknown).

M = male; F = female; SGPT = serum glutamic pyruvic transaminase; SUN = serum urea nitrogen; SAP = serum alkaline phosphatase.

SOURCE: Torkelson et al., 1976.

TABLE 5-4. EFFECTS OF SUBCHRONIC OR CHRONIC ORAL ADMINISTRATION OF CHLOROFORM TO ANIMALS

Species, strain age/weight at start	Sex	No. at start	Vehicle	Dosage	Duration	Response	Reference
Rats, Sprague-Dawley weanling, 101 g M, 94 g F	M,F	20/sex/dose level	drinking water	0, 5, 50, 500, or 2500 ppm in drinking water ad lib. corresponding to intakes[a] of 0, 0.11-0.71, 1.2-1.5, 8.9-14, or 29-55 mg/rat/day The highest dose[a] corresponds to ≈291 mg/kg/day F, 310 mg/kg/day M	90 days, after which 10 rats/group were killed and 10 rats/group observed for additional 90 days	Increased mortality, decreased growth rate, and decreased food intake at highest dose; increased frequency of mild to moderate liver and thyroid lesions at highest dose, including increases in cytoplasmic homogeneity, hepatocyte density, and cytoplasmic volume, vacuolization due to fatty infiltration, some vesiculation of biliary epithelial nuclei, and hyperplasia in livers; and reduced follicle and colloid density, increased epithelial height, some focal collapse of follicles in thyroid; no histopathological effects in kidney, brain, and heart; after the 90-day recovery, the lesions were very mild and similar to those seen in controls	Chu et al., 1982b

TABLE 5-4. (continued)

Species, strain age/weight at start	Sex	No. at start	Vehicle	Dosage	Duration	Response	Reference
Rats, Osborne-Mendel 6 weeks/190 g	M	30/group except 40 ad lib. controls	drinking water	0, 200, 400, 600 900, or 1800 ppm in drinking water ad lib. corresponding to intakes[b] of 0, 20, 38, 57, 81, or 160 mg/kg/day, plus 0 ppm group matched with 1800 ppm group for water consumption	10 rats/group killed at 30, 60, and 90 days of exposure	Dose-related signs of depression during 1st week only; dose-related reduction in water consumption; decreased weight gain in 160 mg/kg group; increased incidence of "hepatosis" in livers of treated rats at 30 and 60 days but not 90 days (not dose-related); no other treatment-related effects on serum clinical chemistry values or urinalysis values, or gross and microscopic appearance of tissues, including kidney.	Jorgenson and Rushbrook, 1980

TABLE 5-4. (continued)

Species, strain age/weight at start	Sex	No. at start	Vehicle	Dosage	Duration	Response	Reference
rats, Osborne-Mendel 52 days/240 g M 175 g F	M,F	50/sex/dose level; 20/ sex-matched controls; ≈99/sex colony controls	corn oil, gavage	M: 0, 90, or 180 mg/kg/day; F: 0, 100, or 200 mg/kg/day (TWA); 5 days/week	78 weeks treatment plus 33 weeks observation	Treated animals had dose-related decrease in survival and weight gain, slight decrease in food consumption, increased severity and incidence of pulmonary lesions characteristic of pneumonia; necrosis of hepatic parenchyma NS in controls, 3/50 low dose M, 4/50 high dose M, 3/49 low dose F, 11/48 high dose F; hyperplasia of urinary bladder epithelium 1/18 control M, 7/45 low dose M, 1/45 high dose M, NS for control F, 6/43 low dose F, 2/40 high dose F; increased splenic hematopoiesis in 1/18 control M, 3/45 low dose M, 6/45 high dose M; both control and treated animals had chronic nephritis; low but statistically significant increased incidence of renal epithelial tumors in treated M (see Carcinogenicity section)	NCI, 1976

TABLE 5-4. (continued)

Species, strain age/weight at start	Sex	No. at start	Vehicle	Dosage	Duration	Response	Reference
rats, Sprague-Dawley NS	M,F	10/sex/dose level	toothpaste, gavage	0, 15, 30, 150, or 410 mg/kg/day 6 days/week	13 weeks	At 410 mg/kg/day, increased liver weight with fatty change and necrosis, gonadal atrophy, increased cellular proliferation in bone marrow; at 150 mg/kg/day, changes less pronounced but effect (NS) on relative liver and kidney weights; presumably no effects at lower dosage levels	Palmer et al., 1979
rats, Sprague-Dawley SPF, 180 to 240 g M 130 to 175 g F	M,F	50/sex/group	toothpaste, gavage	0 or 60 mg/kg/day 6 days/week	80 weeks exposure plus 15 weeks observation	Survival of treated animals slightly better than that of controls (32% treated M, 22% control M, 26% treated F, 14% control females survived to 95 weeks); body weights of treated rats slightly and progressively depressed; intercurrent respiratory and renal disease in all groups; minor histological changes in livers but no evidence of "treatment-related toxic effect" in	Palmer et al., 1979

TABLE 5-4. (continued)

Species, strain age/weight at start	Sex	No. at start	Vehicle	Dosage	Duration	Response	Reference
						livers; decrease ($p<0.01$) in relative liver weight in treated females; no gross or histologic treatment-related changes in brain; possible effect (NS) on incidence of severe glomerulonephritis; decrease in plasma cholinesterase in treated females	
mice, B6C3F1, 6 weeks/19 g	F	30/group except 40 ad lib. controls	drinking water	0, 200, 400, 600, 900, 1800, or 2700 ppm in drinking water ad lib. corresponding to intakes[c] of ≈0, 32, 64, 97, 145, or 290 mg/kg/day; additional 0 ppm group matched with 2700 ppm group for water consumption	10 mice/group killed at 30, 60, and 90 days of exposure	Dose-related signs of depression during first week only; marked reduction in water consumption in higher-dose groups during first 2 weeks; body weight losses ($p<0.05$) in 97, 145, and 290 mg/kg groups and in matched controls during first week; mild hepatic centrilobular fatty change in 64, 97, 145, and 290 mg/kg groups at 30 days, but only in 2 highest dosage groups at 60 and 90 days; increase in liver fat/liverweight	Jorgenson and Rushbrook, 1980

TABLE 5-4. (continued)

Species, strain age/weight at start	Sex	No. at start	Vehicle	Dosage	Duration	Response	Reference
						($p<0.05$) for 290 mg/kg group at all 3 periods; no other treatment-related changes in serum enzyme levels, urinalysis values, or gross or microscopic appearance of tissues including kidney	
mice, B6C3F1 35 days/18g M, 17 g F	M,F	50/sex/dose level; 20/sex matched controls	corn oil, gavage	M: 0, 138, or 227 mg/kg/day; F: 0, 238, or 447 mg/kg/day; 5 days/week	78 weeks treatment plus 14 to 15 weeks observation	Survival decreased in high dose F, unaffected in other treated groups; high incidences of hepatocellular carcinoma in treated mice (see Carcinogenicity section); renal inflammation in 10/18 control M, 2/50 low dose M, 1/50 high dose M	NCI, 1976
mice, Schofield	M,F	10/sex per dosage	toothpaste, gavage	0, 60, 150, or 425 mg/kg/day; 6 days/week	6 weeks	At 425 mg/kg, 100% mortality; at 150 mg/kg, 8/10 M died and weight gain of F markedly retarded; at 60 mg/kg, weight gain of both sexes moderately retarded; no other observations mentioned	Roe et al., 1979

TABLE 5-4. (continued)

Species, strain age/weight at start	Sex	No. at start	Vehicle	Dosage	Duration	Response	Reference
mice, ICI (expt. 1) ICI (SPF) (expt. 2) ICI, CBA, C57BL, CF/1 (expt. 3) ≤10 weeks old	expt. 1: M,F expt. 2 and 3: M treated and control, plus some F control	treated: 52/sex/dose level; control: 52 to 206/sex/strain/vehicle plus untreated	toothpaste in all 3 expt. for all strains and sexes plus arachis oil in expt. 3 for ICI, gavage	0, 17 (expt.1), or 60 mg/kg/day, 6 days/week	80 weeks treatment; 16 to 24 weeks observation according to numbers of survivors	Survival generally better in 60 mg/kg groups than in controls except for CF/1 animals or when chloroform given in arachis oil; slight retardation of weight gain in 60 mg/kg groups; no effect on hematologic values (tested in expt. 2 only); no treatment-related adverse effect on liver or other tissues except in kidneys as follows: 60 mg/kg in toothpaste - increased incidence of moderate to severe renal changes ($p<0.001$) in CBA and CF/1 M, 60 mg/kg in oil-increased incidence of moderate to severe kidney disease ($p<0.05$) in ICI M; increased incidence of benign and malignant kidney tumors in ICI M treated with 60 mg/kg in toothpaste or oil (see Carcinogenicity section)	Roe et al., 1979

TABLE 5-4. (continued)

Species, strain age/weight at start	Sex	No. at start	Vehicle	Dosage	Duration	Response	Reference
dogs, beagle 18 to 24 weeks 7 to 8 g	M,F	1/sex/dose level for 90 and 120 mg/kg; 2/sex/dose level for lower dosages	toothpaste in gelatin capsule, orally	30, 45, 60, 90, or 120 mg/kg/day, 7 days/week	13 weeks for 30 and 45 mg/kg, 18 weeks for 60 mg/kg, 12 weeks for 90 and 120 mg/kg	No deaths; occasional vomiting; marked weight loss in all dogs and poor general condition in some at 60 mg/kg or higher; apparent suppression of appetite initially at all dosages and throughout at 60 mg/kg and higher; jaundice and increased SAP, SGOT, SGPT, bilirubin, and ICD values in male at 120 mg/kg; increased SGPT values in 4/4 and increased SAP and SGOT values in 2/4 at 60 mg/kg; hepatocyte enlargement and vacuolation with fat deposition at 60 mg/kg and higher; discoloration of liver, increased liver weight, and slight fat deposition in hepatocytes at 45 mg/kg; no effect on any of these clinical chemistry or histological parameters at 30 mg/kg	Heywood et al., 1979

TABLE 5-4. (continued)

Species, strain age/weight at start	Sex	No. at start	Vehicle	Dosage	Duration	Response	Reference
dogs, beagle, 8 to 24 weeks, 7 to 8 kg	M,F	8/sex/dose level; 16/sex vehicle controls; plus other controls	toothpaste in gelatin capsule, orally	0, 15, or 30 mg/kg/day; 6 days/week	7.5 years treatment plus 20 to 24 weeks observation	No effect on survival, growth, organ weights, hematologic or urinalysis values (checked at intervals throughout); moderate dose-related elevation of SGPT reaching peak in sixth year of study, reverting to normal levels after treatment discontinued; other serum enzyme indicators of hepatic damage (checked during the latter portion of the study) followed pattern similar to SGPT, but BSP retention and ICD values were unaffected; aggregation of vacuolated histiocytes ("fatty cysts") in livers of all groups but cysts were larger and more numerous in treated dogs and persisted after treatment ended; fat deposition affected more renal glomeruli in 30 mg/kg group than in other groups	Heywood et al., 1979

TABLE 5-4. (continued)

Species, strain age/weight at start	Sex	No. at start	Vehicle	Dosage	Duration	Response	Reference

[a] Calculated by Chu et al. (1982b) by multiplying the fluid intake volume by the concentration of chloroform.
[b] Calculated by Jorgenson and Rushbrook (1980) from measured average body weights and water consumption.
[c] Calculated from Jorgenson and Rushbrook's statement that the mice had actual intake levels of from 148 to 175% of the intended levels of 20, 40, 60, 90, 180, and 270 mg/kg/day.
[d] Growth rate data were given only for the highest dose and mg/kg/day were calculated from this information by taking average weights over the 90-day period of exposure.

SPF = specific pathogen-free; SGPT = serum glutamic-pyruvic transaminase; SAP = serum alkaline phosphatase; BSP = bromsulphthalein;
ICD = isocitric dehydrogenase (serum)

Purity of the chloroform samples used in all these studies was generally high and is discussed in the section on Carcinogenicity.

M = male; F = female; TWA = time-weighted average dose for days on which chemical was administered; NS = Not specified.

(Heywood et al., 1979), and (3) renal damage in male mice of some sensitive strains (Roe et al., 1979), and in dogs (Heywood et al., 1979). In addition to hepatic damage, ingestion of ≃300 mg/kg/day of chloroform produced thyroid lesions in rats (Chu et al., 1982b). In one study, a decreased incidence of renal inflammation occurred in male mice treated with chloroform at 138 or 227 mg/kg/day, 5 days/week (NCI, 1976). A similar effect may have occurred in rats (Palmer et al., 1979), but the authors did not specify whether the effect of chloroform was to increase or decrease the incidence of intercurrent renal disease. The NCI (1976) report stated that hepatic necrosis, hyperplasia of the urinary bladder epithelium, and increased splenic hematopoiesis in rats may have been related to chloroform treatment, but incidences in controls for some of these effects were not reported and the data, shown in Table 5-4, are difficult to interpret. Many of the studies summarized in Table 5-4 were at least partially designed as investigations of carcinogenicity and, hence, are also discussed in the carcinogenicity section of this document; some experimental details are discussed more fully in that section.

From the data presented in Table 5-4, it appears that rats and mice can tolerate higher daily intakes of chloroform when it is given in their drinking water or in a toothpaste base (by gavage) than they can when the chemical is administered in corn or arachis oil (by gavage). In the subchronic study of Jorgenson and Rushbrook (1980), rats and mice appeared to adapt to low levels of chloroform intake (up to ≃100 mg/kg/day); signs of depression and mild hepatic damage that occurred initially had disappeared by 90 days of treatment. Elevated indices of liver damage (e.g., SGPT levels) in dogs chronically exposed to chloroform reverted to "normal" after treatment was discontinued, although histological changes persisted.

Similarly, the mild liver and thyroid lesions seen in rats exposed to high doses of chloroform via their drinking water for 90 days were no longer apparent in rats allowed to recover for an additional 90 days (Chu et al., 1982b).

5.3. INVESTIGATION OF TARGET ORGAN TOXICITY IN EXPERIMENTAL ANIMALS

Chloroform is a hepatic and renal toxicant in a variety of animal species including man. In mice, however, nephrotoxicity occurs only in males and not in females, although the hepatotoxicity is similar in both sexes. This sex difference in mice appears to be androgen-related. Condie et al. (1983) have compared the renal and hepatoxicity of chloroform with other halomethanes in male mice (CD-1) after daily oral dosing in corn oil for 14 consecutive days at three dose levels. Toxicity was evaluated by measuring change in total body weight, uptake of p-aminohippurate (PAH) into renal cortical slices, blood urea nitrogen, serum creatinine, serum GPT, and by performing histopathologic examination of liver and kidney tissues. Chloroform was the most potent of the series followed by dibromochloromethane, bromodichloromethane, bromoform, and methylene chloride with the least organ toxicity.

5.3.1. Hepatotoxicity

An extensive review of the early literature dealing with chloroform-induced liver damage by von Oettingen (1964) notes studies beginning in 1891. More recently, Groger and Grey (1979) summarized reports describing chloroform-induced liver hepatotoxicity as follows: typical effects of chloroform on liver cells are extensive vacuolization, disappearance of glycogen, fatty degeneration, swelling, and necrosis, all starting in the centrilobular areas. There is also often hemorrhaging into the parenchyma and infiltration of polymorphonuclear cells and monocytes.

Electron-microscopic observations of liver parenchymal cells from chloroform-intoxicated rats as carried out by Scholler (1966, 1967) revealed deposition of lipid droplets in the cytoplasm, partial destruction of the mitochondrial matrix, proliferation of smooth endoplasmic reticulum, and swelling of the rough endoplasmic reticulum with detachment of ribosomes.

Kylin et al. (1963) conducted a study of the hepatotoxic effects of inhaled trichloroethylene, tetrachloroethylene, and chloroform in mice with the objective of finding the lowest (individual) concentration of the substances producing signs of liver damage after a single 4-hour exposure period. Histological examination showed that concentrations of these three agents at 100 ppm caused moderate fatty infiltration in mice killed 1 day after exposure. At ≥200 ppm, the extent of the alteration increased with concentration and was more pronounced after 1 day than 3. Thus, judging from the histological picture, the smallest concentrations (ppm) of the different agents to produce more severe alterations in the exposed group than in the controls were as follows:

	Trichloroethylene (ppm)	Tetrachloroethylene (ppm)	Chloroform (ppm)
1 day after exposure	1600-3200	<200	<100
3 days after exposure	>3200	200 to 400	100 to 200

On this basis, the hepatotoxic effects of trichloroethylene, tetrachloroethylene, and chloroform are in the approximate ratios 1:10:20. The amount of liver fat was raised at 400 ppm of chloroform. A third indicator of liver toxicity was an increase in serum ornithine carbamyl transferase (S-OCT) activity at 24 hours in animals exposed to 200, 400, and 800 ppm of chloroform.

A study of the effect of oral doses of chloroform on the extent of liver damage in white mice ("of a Swiss strain") was conducted by Jones et al. (1958). Minimal changes characterized by midzonal fatty infiltration were observed 72 hours after the administration of 30 mg (0.02 ml)/kg. When the dose was increased to 133 mg (0.09 ml)/kg, a massive fatty infiltration of the total liver lobule was found. At a level of 355 mg (0.24 ml)/kg, massive fatty infiltraton occurred along with severe central lobular necrosis. Information on the hepatotoxicity of long-term chloroform administration has been presented in the sections on Effects of Chronic Exposure to Chloroform. Inhalation exposure of rats to 25, 50, or 85 ppm chloroform for 7 hours/day, 5 days/week for 6 months produced centrilobular granular degeneration and focal necrosis in their livers. In subchronic studies, ingestion of up to ≈100 mg/kg/day of chloroform produced mild, transient histological changes in the livers of rats and mice (Jorgenson and Rushbrook, 1980), ingestion of 145 or 190 mg/kg/day produced fatty change in the livers of mice (Jorgenson and Rushbrook, 1980), and administration of 410 mg/kg/day by gavage produced fatty change and necrosis in the livers of rats. Dogs treated subchronically with 45 mg/kg/day by the oral route had histological evidence of slight hepatic fatty change, with increasingly severe changes noted at dosages of 60 and 120 mg/kg/day.

In chronic oral studies, rats had minor histological change in their livers and a decrease in relative liver weights when given 60 mg/kg/day of chloroform, 6 days/week, while the livers of mice were unaffected at this dosage. Dogs had some evidence of liver damage (clinical chemistry parameters) and an increase in the number and size of fatty cysts in their lifetime when administered 15 or 30 mg/kg/day orally for 6 days/week. The mechanism by which chloroform exerts its hepatotoxic effects has been widely investigated and efforts have been made to identify the responsible metabolite(s).

As long ago as 1928, it was suspected that the liver damage induced by chloroform may be due not only to the chemical itself, but might be caused by a degradation product (Lucas, 1928). Chloroform has been determined to be metabolized before being excreted (Butler, 1961; Paul and Rubenstein, 1963; Van Dyke et al., 1964; Reid and Krishna, 1973). These studies indicate that the tissue necrosis induced by chloroform is associated with the covalent binding of toxic metabolites and alkylation of tissue proteins. Autoradiograms have revealed that this binding occurs predominantly in the necrotic areas (Illet et al., 1973). McLean (1970) has shown that pretreatment of rats with phenobarbital (a microsomal enzyme-inducing agent) greatly enhances the lethality of chloroform.

Brown et al. (1974) proposed a mechanism of chloroform hepatotoxicity implicating a free radical metabolite which can react with glutathione (GSH) (a tripeptide which protects against hepatotoxicity), diminishing GSH levels in the liver. According to this hypothesis, once GSH levels are depleted, further metabolism would result in the reaction of the metabolite with microsomal protein, and hence, necrosis. This proposal was based on observations of phenobarbital pretreated rats anesthetized with chloroform, in which hepatotoxicity was enhanced and GSH levels were decreased by the induction of microsomal enzymes. Covalent binding of chloroform metabolites to microsomal proteins *in vitro* was also enhanced by enzyme induction, an effect prevented by GSH. Similar findings were reported in mice by Ilett et al. (1973), who found severe chloroform-induced centrilobular necrosis in phenobarbital pretreated mice, but only slight centrilobular damage in mice exposed only to chloroform.

Thus, the hepatotoxicity of chloroform appears to depend on (1) the rate of its biotransformation to produce reactive metabolite(s), and (2) the amount of GSH available to conjugate with and thus inactivate the metabolite(s).

The role of GSH in chloroform-induced hepatotoxicity was further studied by Docks and Krishna (1976), who found that only those doses of chloroform that decreased liver GSH caused liver necrosis when administered to phenobarbital pretreated rats.

More recently, Ekström et al. (1982) studied the mechanism of GSH depletion by chloroform in rats pretreated with phenobarbital. The synthesis of GSH proceeds via two enzymatic steps, the first of which is rate limiting:

$$\text{glutamate} + \text{cysteine} \xrightarrow[\text{synthetase}]{\gamma\text{-glutamyl-cysteine}} \text{dipeptide}$$

In the presence of glycine, the reaction continues via GSH synthetase to produce GSH. When the soluble fraction from livers of rats sacrificed at various times after chloroform exposure was incubated in the presence of these amino acids, it was found that GSH synthesis was inhibited within 4-6 hours, while liver necrosis was evident only after 6 hours. When glycine was eliminated from the initial part of the incubation, the dipeptide accumulated, but at a lower rate in the presence of chloroform than in its absence. Later addition of glycine resulted in GSH synthesis at a rate similar to control values. Thus, it appears that chloroform, or rather a reactive metabolite, inhibited GSH synthesis at the rate limiting step (i.e., the formation of dipeptide by γ-glutamyl-cysteine synthetase).

Savage et al. (1982) found that rats treated with chloroform for 1 to 7 days showed a dose-dependent increase in hepatic ornithine decarboxylase (ODC), with a threshold at 100 mg/kg body weight. Females were 2 to 4 times more susceptible than males, and rats dosed from 7 days showed a decline in ODC susceptibility. Chloroform, rather than increasing the activity of renal ODC, resulted in a 35% reduction. The induction by chloroform of hepatic ODC might be associated with regenerative hyperplasia, while the renal carcinogenicity of chloroform could not be associated with ODC induction. The same researchers have reported more recently that chloroform's role in inducing liver and renal ODC activity does not appear to be associated with a regenerative response (Pereira et al., 1984).

The biotransformation of chloroform (as discussed in Chapter 4) depends on the activity of the microsomal drug metabolizing enzymes. Substances that induce these enzymes were shown to, indeed, enhance the hepatotoxicity of chloroform, as evidenced by increased serum glutamic-pyruvic transaminase (SGPT) levels and decreased hepatic glucose-6-phosphatase activity (Lavigne and Marchand, 1974). An inhibitor of the drug metabolizing enzymes SKF-525A, however, while increasing the excretion of ^{14}C-carbon monoxide in rats administered ^{14}C-labelled chloroform, failed to diminish the hepatotoxicity of chloroform, leading these authors to conclude that factors other than metabolism may be involved.

McMartin et al. (1981) demonstrated that altering the cytochrome P-450 concentrations in the livers of chloroform-exposed rats also altered the hepatotoxicity, as measured by the incidence of hepatic lesions and by serum alanine aminotransferase activities. Both fasting and phenobarbital pretreatment increased the cytochrome P-450 content and liver damage, while cadmium produced the opposite effect.

Theories of chloroform hepatotoxicity involve the formation of reactive intermediates by liver enzymes. How these intermediates exert their hepatotoxic effect has been the subject of several studies. It has been suggested by Masuda et al. (1980) that, based on the chloroform-induced indices of hepatotoxicity of decreased microsomal glucose-6-phosphatase activity and cytochrome P-450 content with increased hepatic malondialdehyde levels, the lipid peroxidation hypothesis proposed for carbon tetrachloride may also apply to the case of chloroform. Qualitative and mechanistic differences of hepatotoxicity between the two chemicals were noted, however.

The interactive hepatotoxicity of chloroform and carbon tetrachloride was studied by Harris et al. (1982), who found that neither chemical alone given at subthreshold dose altered SGPT activity, hepatic triglyceride content, or hepatic calcium content. However, when given together, chloroform and carbon tetrachloride increased the toxic response in rats. Administration of either or both chemicals had no effect on GSH levels or conjugated diene formation, but ethane expiration was increased in rats given both chemicals. Diene conjugation and ethane expiration are indices of lipid peroxidation. Histopathological changes were more severe from the combination than from either chemical alone. Although the mechanism of the hepatotoxic interaction between chloroform and carbon tetrachloride is unclear, the authors suggested that there might be a combined effect of phosgene formation and lipid peroxidation initiation.

It should be noted that the prevailing theories implicate phosgene as the major metabolite responsible for chloroform hepatotoxicity (Reynolds and Yee, 1967; Sipes et al., 1977; Mansuy et al., 1977; Pohl et al., 1977). Other potential toxic metabolites discussed by Pohl (1979) in a review of this subject are a trichloromethyl radical and dichlorocarbene; however, they are considered less important than phosgene in this regard.

A study by Stevens and Anders (1981) supports the phosgene-mediated mechanism. The time course of changes in SGPT levels and covalent binding of ^{14}C to proteins was examined in microsomal and soluble fractions from phenobarbital-pretreated rats sacrificed at various times after chloroform or ^{14}C-chloroform administration. It was found that ^{14}C binding was maximal at 6 hours while indices of liver damage peaked at 18 hours after chloroform exposure. Further experiments were performed in which diethyl maleate (which depletes GSH depletor) treatment caused increased ^{14}C-binding to soluble and microsomal fractions and increased SGPT levels, perhaps by inhibiting the metabolism of phosgene to carbon monoxide or stable conjugates. Cysteine, which reacts with phosgene to produce 2-oxothiazolidine-4-carboxylic acid, had a protective effect. Diethyl maleate also diminished, but did not eliminate the deuterium isotope effect on the GSH dependent chloroform metabolism to carbon monoxide, which would be expected if carbon monoxide formation occurred subsequent to phosgene production. Thus, the hepatotoxicity of chloroform can be altered by altering the various reaction pathways of phosgene, strongly indicating that phosgene is the toxic intermediate.

From the above discussion, it appears that to be hepatotoxic, chloroform must first be metabolized by microsomal drug metabolizing enzymes to an active intermediate, probably phosgene, which in turn can react by various pathways, depending on GSH levels. One pathway is the covalent binding to liver proteins, resulting in necrotic lesions. Lavigne et al. (1983) have pointed out that circadian rhythms may also influence chloroform-induced hepatotoxicity. These investigators determined the diurnal variation of chloroform-induced hepatotoxicity in Sprague-Dawley rats. Animals were dosed at 9:00, 13:00, 17:00, 21:00 or 03.00 hr (i.p., 0.5 ml/kg) and sacrificed 4 hr after each dosing. Measures of toxicity included serum GPT, GOT, LDH and liver glucose-6-phosphatase. Toxicity was minimum after 9:00 hr dosing and maximal 12 hr later at 21:00 hr dosing. Prior 16 hr food deprivation increased the level of toxicity. Lavigne et al. suggest that a correlation exists between the diurnality of chloroform-induced hepatotoxicity and drug metabolizing enzyme activity, which in rats is higher in the evening than in the morning.

5.3.2. Nephrotoxicity

As noted by Watrous and Plaa (1972), the extensive body of research on the hepatotoxicity of halogenated hydrocarbons has tended to overshadow the fact that some of these agents are also nephrotoxic. Earlier reports of chloroform nephrotoxicity include those of Heller and Smirk (1932), who found that rats anesthetized with chloroform showed a diminished ability to excrete a water load given prior to anesthesia, and Knocher and Mandelstam (1944), who noted that chloroform injection produced a fatty infiltration of the kidney.

Renal necrosis produced by the oral administration of chloroform was described by Eschenbrenner and Miller (1945a). The necrosis, observed only in male mice, involved portions of both proximal and distal convoluted tubules. The nuclei of the epithelial cells were often absent or fragmented and the cytoplasm was coarsely granular and deeply eosinophilic. The glomeruli and collecting tubules appeared normal.

Sex and strain differences in the sensitivity of mice to chloroform nephrotoxicity were further studied by Deringer et al. (1953). Exposure of strain C3H mice to air containing $\simeq 5$ mg/L of chloroform for 1, 2, or 3 hours resulted in lesions of the kidneys of all of the males but in none of the females. In animals dying within 1 day after exposure, epithelium of the proximal tubules and portions of the distal tubules were generally necrotic. The lumens of those segments of tubules were dilated. The glomeruli were relatively unaffected. The mice dying or sacrificed at later time intervals exhibited calcification in the necrotic area.

Similar lesions were found in males of strains C3H, C3Hf, A, and HR. However, strains C57BL, C57BR/cd, C57L, and ST were resistant to chloroform-induced nephrotoxicity.

Comparable results were reported by Krus and Zaleska-Rutczynski (1970). Subcutaneous administration of chloroform to C3H/He male mice resulted in renal tubular necrosis, with death ensuing 4-9 days later. The lesions were calcified with no evidence of regeneration. Female mice of this strain, males and females of the C57BL/6JN and BN strains, and F_1 generation males of the cross of female C3H/He with male C57BL/6JN mice survived the administration of chloroform (0.1 ml of 0.05 g chloroform in 1 ml ethyl laureate). Additional studies were performed with males and females of this F_1 generation and the resistant BN strain, in which animals were sacrificed at various times after chloroform administration. All mice survived, while all female mice were resistant, showing no kidney lesions at any time in the experiment. Renal damage was morphologically apparent in all male mice by 12 hours, but regeneration developed by day 4 and continued until the end of the experiment. It was concluded that although all male mice had tubular lesions, the ones surviving had tubules that did not calcify and a large degree of renal regeneration.

Several investigators have studied the influence of testosterone on chloroform-induced renal damage. Eschenbrenner and Miller (1945b) performed experiments in which they saw extensive necrosis of portions of the proximal and distal renal tubules in normal male and in testosterone-treated castrated male mice following the acute oral administration of chloroform. However, no necrosis was found after chloroform was administered to female mice or castrated male mice. Continuing this line of investigation, Culliford and Hewitt (1957) reported the following results:

1) Adult male mice of two strains (CBA and WH) developed extensive necrosis of the renal tubules after exposure to low concentrations of chloroform vapor (7-10 mg/L for 2 hours). Adult females showed no renal damage after equivalent exposure.
2) Adult females became fully susceptible to necrosis after treatment with androgens. The susceptibility of males was greatly reduced by treatment with estrogens.
3) Castration removed the susceptibility of the males of one strain, but did not completely remove it in another. The residual susceptibility of castrates was abolished by adrenalectomy.
4) Male mice under 11 days old were not susceptible to necrosis even after massive doses of androgen. Between 11 and 30 days, they were susceptible if given androgen. Thereafter, they became spontaneously susceptible.
5) Liver damage occurred in nearly all exposed mice and was not correlated with sex hormone status.
6) Susceptibility could be induced in gonadectomized mice by methyl testosterone, testosterone propionate, dehydroepiandrosterone, progesterone, and large doses of cortisone acetate.

Hill (1978) also performed experiments demonstrating similar strain and sex differences in chloroform-induced renal toxicity. The renal toxicity of a fixed oral dose of chloroform to castrated male mice was increased with increasing doses of administered testosterone. Plasma levels of testosterone in resistant strains tended to be lower than levels in susceptible strains. Hill (1978) conjectured that a testosterone may act by sensitizing the renal proximal convoluted tubules to chloroform through a testosterone receptor mechanism. Eschenbrenner and Miller (1945b), however, linked susceptibility to the nephrotoxic action of chloroform to differences in kidney morphology and physiology induced by testosterone.

Information on the nephrotoxicity of long-term chloroform administration has been presented in the section on Effects of Chronic Exposure to Chloroform. Inhalation of 25, 50, or 85 ppm of chloroform 7 hours/day, 5 days/week for 6 months produced cloudy swelling of the renal tubular epithelium in rats. Male mice of certain sensitive strains had increased incidences of moderate to severe renal disease when treated orally with chloroform at a dosage of 60 mg/kg/day, 6 days/week in a chronic study (Roe et al., 1979). Chronic oral administration of 30 mg/kg/day of chloroform, 6 days/week, to dogs produced an increase in the numbers of renal glomeruli affected by fat deposition (Heywood et al., 1979).

The mechanism of the nephrotoxicity of chloroform has been extensively studied in the last few years. Earlier, the possibility that hepatotoxic metabolites produced in the liver might be transported via the circulation to the kidney to produce nephrotoxic effects had been considered (Ilett et al., 1973). Recent studies have shown that chloroform is metabolized within the kidney and the nephrotoxic effects of chloroform are the resultant of that metabolism. The evidence can be summarized as follows:

1) It has been known that kidney slices from rats (Paul and Rubinstein, 1963) and more recently from mice (Smith and Hook, 1983) can metabolize chloroform to CO_2, which is a known degradation producer of phosgene (Pohl et al., 1980). Smith and Hook (1983, 1984) have further shown that ^{14}C-labelled chloroform is metabolized by renal cortical microsomes from male ICR mice (but to a markedly lesser extent by renal microsomes from female mice) to $^{14}CO_2$ and to reactive metabolites that covalently bind to microsomal protein and lipids.

renal metabolism, although 3- to 5-fold less active than hepatic microsomal metabolism, also required NADPH and O_2. P_{450} binding of chloroform, as in the liver, was Type 1 and was inhibited by CO, while addition of GSH decreased $^{14}CO_2$ production and covalent binding. Pohl et al. (1984) found that renal cortical homogenates from chloroform sensitive ICR male mice (10-fold more active than female mice) fortified with NADPH, O_2 and cysteine metabolized chloroform to phosgene, which was detected by HPLC as the cysteine derivative, 2-oxothiazolidine-4-carboxylic acid (OTZ). P_{450} in both microsomal and mitochondrial fractions (microsomes 20x more active) catalyzed chloroform to phosgene. Cleavage of the C-H bond of chloroform was the rate-limiting step since chloroform metabolism was 3x more rapid than with the deuterated analog $CDCl_3$. Branchflower et al. (1984) have reported entirely similar findings; after incubation of kidney homogenates with GSH they identified OTZ and OTZG (N-(2-oxothiazolidine-4-carboxyl)-glycine) formed after initial reaction of phosgene with glutathione. The intermediate diglutathionyl dithiocarbonate (GSCOSG) was not identifiable, but it was found to be rapidly converted to OTCG and OTZ by kidney γ-glutamyl transpeptidase and cystinyl glycinase. Figure 5-1 shows the probable pathways of metabolisim of chloroform in the kidney. OTZ and GSCOSG are not nephrotoxic either in vivo or in vitro (as assessed by effects on PAH accumulation by renal cortical slices). The pathway therefore represents a detoxification mechanism for phosgene, the reactive species formed in the kidney by P_{450} mediated oxidative metabolism of chloroform.

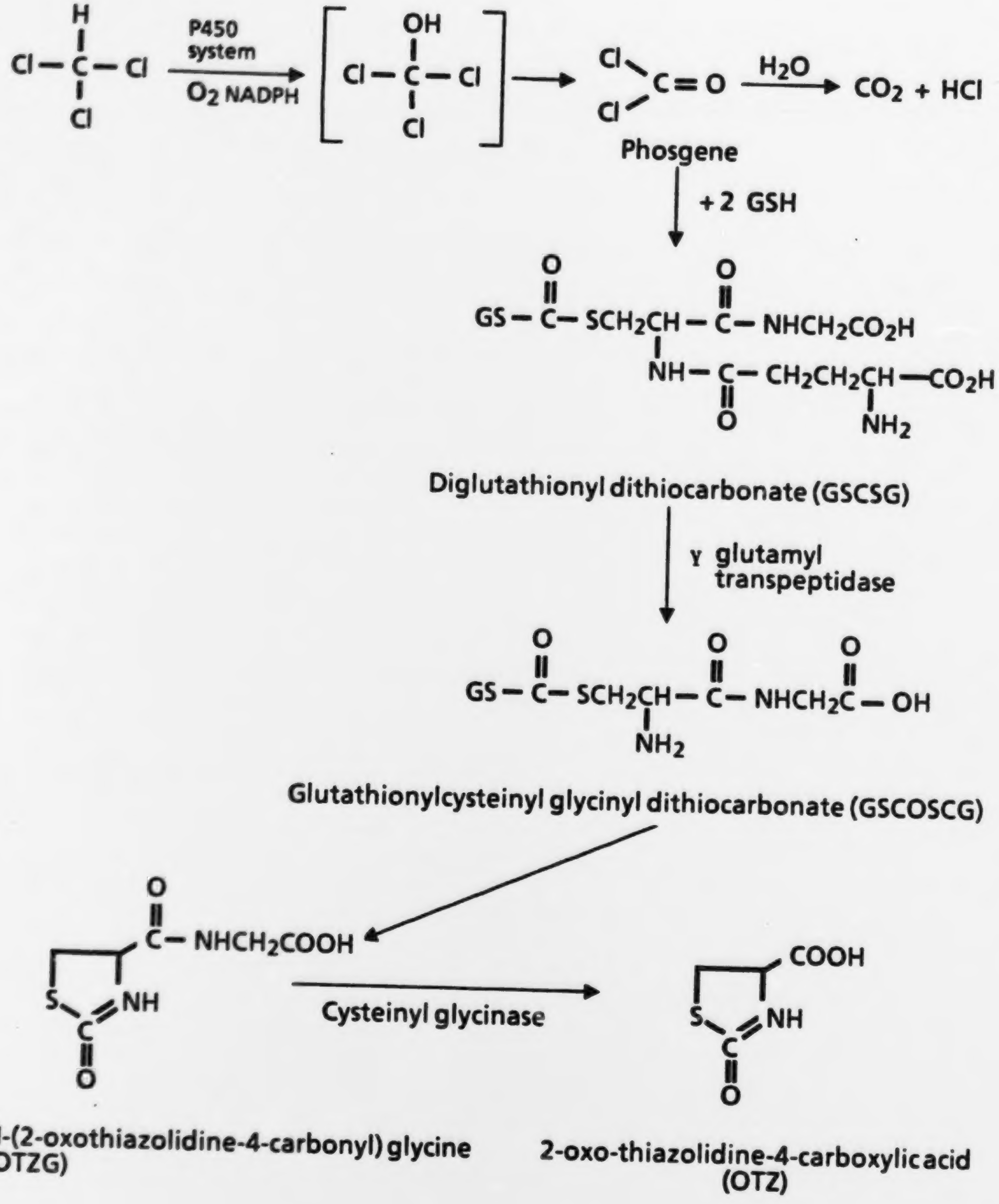
P450 system
O2 NADPH
Phosgene
H2O
CO2 + HCl
+2 GSH
Diglutathionyl dithiocarbonate (GSCSG)
γ glutamyl transpeptidase
Glutathionylcysteinyl glycinyl dithiocarbonate (GSCOSCG)
Cysteinyl glycinase
N-(2-oxothiazolidine-4-carbonyl) glycine (OTZG)
2-oxo-thiazolidine-4-carboxylic acid (OTZ)

Figure 5-1. Probable pathways of metabolism of chloroform in the kidney (from Branchflower et al., 1984).

2) The administration of chloroform to mice or rats produces a depletion of kidney GSH, which indicates that phosgene is formed as a metabolite in vivo (Kluwe and Hook, 1981), while deuterated chloroform ($CDCl_3$) depletes renal GSH to a lesser extent and is less toxic (Branchflower et al., 1984). Similarly GSH added to renal homogenates or microsomes decreases covalent binding from chloroform metabolism (Smith and Hook, 1984).

3) The capacity of the kidney to metabolize chloroform to phosgene correlates with strain and sex differences in susceptibility to chloroform-induced nephrotoxicity. Ahmadizadeh et al. (1984a) investigated the possibility that morphological differences in cell type between sexes and strains of mice may account for differences in renal toxicity to chloroform. However, these workers found that renal lesions induced by chloroform in male strains of mice were predominantly located in proximal convoluted tubule epithelial cells; no histopathological changes were observed in cuboidal cells of Bowman's capsule, although differences in cell type occurs between sexes as well as strain in capsular epithelium. They concluded that these morphological differences between sexes and strains probably are not responsible for the pathophysiological difference seen with chloroform, but rather a common factor such as testosterone effect on metabolism and on morphology. Smith et al. (1983) explored the time course of chloroform toxicity in male and female ICR mice. They observed a rapid nephrotoxic effect in male mice as early as 2 hr after s.c. injections of chloroform (250 µl/kg). Renal cortical slices from treated male mice exhibited decreased uptake and accumulation of PAH and TEA, paralleled by a decrease of glutathione

tissue content. Cytopathologic changes appeared 5 hr after chloroform treatment. Female mice showed no evidence of renal toxicity, even with the administration of diethylmaleate to deplete tissue glutathione. On the other hand, hepatotoxicity was similar in male and female rats and occurred with a depletion of hepatic glutathione. These workers concluded that the temporal events in liver and kidney toxicity suggested that chloroform was metabolized by both liver and kidney in male mice but only in the liver of female mice. Smith et al. (1983) provided further evidence from _in_ _vitro_ studies. Renal cortical slices from ICR mice incubated with chloroform resulted in a decrase of PAH or TEA uptake and accumulation when the slices were from male mice but not female mice. Deuterated chloroform ($CDCl_3$) had a lesser effect than chloroform. Furthermore, the chloroform toxicity was inhibited by CO or by incubation of the slices at 0°C. Conversely, renal slice toxicity (as measured by PAH or TEA uptake) was increased by pretreatment of the male mouse with diethylmaleate, a depletor of tissue GSH. Smith et al. (1984) also investigated the effect of sex hormonal status on chloroform nephrotoxicity and renal mixed function oxidases in ICR mice. They found that renal microsomal content of cytochrome P_{450} was greater (5-fold) in kidneys from male mice versus female mice. Similarly cytotochrome b_5 content was greater (2-fold) and P_{450} mixed function oxidase activities (ethoxycoumarin O-deethylase) was greater (3- to 5-fold). Treatment of female mice with testosterone increased renal P_{450} content and activity to the male mouse level, while castration of male mice reduced P_{450} content and activity to that of female mice. Smith and Hook (1984) showed further that renal cortical microsomes from ICR male mice, but not female mice, metabolized ^{14}C-chloroform to reactive metabolites that covalently bind to protein and lipid. These studies are consistent with the concept that chloroform is metabolized by cytochrome P_{450} mechanisms in the kidney and the resulting sex difference of renal metabolic lesions is correlated with a sex difference in renal metabolic capacity to metabolize chloroform. Pohn et al. (1984) have also provided strong experimental evidence for this concept. They found that kidney homogenates of sensitive male DBA/2J mice metabolized chloroform to phosgene 2x more rapidly than less sensitive C57BL/6J strain, and kidney homogenates from male IRC sensitive strain metabolized chloroform to phosgene 10x more rapidly than homogenates from female mice of this strain. Furthermore treatment of IRC female mice with testosterone induced in their kidney homogenates the ability to metabolize chloroform to phosgene to the same level of male mice.

4) Uptake hepatic metabolism and hepatotoxicity of chloroform, potentiation of chloroform toxicity by P_{450} inducers, example phenobarbital, cannot be demonstrated in rat or mouse kidneys, which are not inducible by phenobarbital (McMartin et al., 1981; Ahmadizadeh et al., 1984b). Similarly, inhibitors of P_{450} metabolizing system, SKF-525A, piperony butoxide, etc., do not reduce the nephrotoxicity of chloroform in sensitive mice (Kluwe and Hook, 1981; Lavigne and Marchand, 1974). Ahmadizadeh et al. (1984b) pretreated subchronically the chloroform renal-sensitive DBA/2J strain of male mice and the relatively resistant C57BL/6J male strain with P_{450} inducers phenobarbital, β-naphthoflavone, and

polybrominated biphenyl (PBBs). None of these inducers increased renal toxicity in DBA/2J mice, although all increased hepatic toxicity. However, PBBs increased both hepatic and renal toxicity and induced P_{450} systems in the resistant C57BL/6J strain (Ahmadizadeh et al., 1984b; Kluwe and Hook, 1978). McMartin et al. (1981) have also shown that fasting increased P_{450} concentrations in both liver and kidney of rats, and chloroform-induced damage was enhanced in both organs of fasted animals. Bailie et al. (1984) have recently shown potentiation of chloroform metabolism and toxicity occurs in rabbit kidneys after pretreatment with phenobarbital. Phenobarbital is a known inducer of the P_{450} metabolizing system in rabbit kidneys. Renal cortical slices from phenobarbital-treated rabbits incubated with chloroform exhibited greater chloroform dose-related decreases in uptake and accumulation of PAH and TEA than non-phenobarbital treated controls. Covalent binding from ^{14}C-chloroform metabolism was enhanced 5-fold in renal slices and microsomes prepared from phenobarbital-treated rabbits. Addition of cysteine to the microsomal incubations resulted in decreased binding, but an increase of ^{14}C-OTZ, a measure of phosgene production. The lack of correlation in mice and rats, but a good correlation in rabbits, between hepatotoxicity and nephrotoxicity of chloroform following pretreatment with enzyme inducers supports the concept that the nephrotoxic metabolite(s) of chloroform are generated in the kidney and do not derive from liver metabolism.

The mechanism underlying the genetically determined strain and sex differences for chloroform metabolism and nephrotoxicity in the rodent remain incompletely resolved. Pohl et al. (1984), noting that testosterone is known to elicit a variety of inductive effects in the mouse kidney, which include hypertrophy and augmentation of many enzyme activities, including the metabolism of chloroform to phosgene, suggest plasma testosterone levels (3,5-fold higher in sensitive DBA/2J male mice than in resistant C57BL/6J male mice) may be responsible, at least in part, for both sex and strain differences in sensitivity to chloroform-induced nephrotoxicity.

5.4. FACTORS MODIFYING THE TOXICITY OF CHLOROFORM

From the preceding discussion, the severity of toxic effects induced by a given amount of chloroform is influenced by alterations of microsomal enzyme activity or hepatic GSH levels. Thus, many factors including exposure to other chemicals alter chloroform toxicity by affecting these parameters or acting through other mechanisms. These factors are of interest because they fall into categories of nutritional status and accidental or intentional chemical exposure by humans. As an example of action and interactions of these categories, Sato and Nakajima (1984) have recently reviewed the experimental evidence for dietary carbohydrate and ethanol-induced alteration of the metabolism and toxicity of chemical substances with chloroform and carbon tetrachloride as illustrative compounds. Sato and Nakajima put forth three main conclusions:

1) Food deprivation causes a 2- to 3-fold increase in the metabolism of various chemicals in rat liver, such as chloroform and carbon tetrachloride. The lack of carbohydrate rather than the lack of protein or fat accompanying food deprivation is primarily responsible for the increase.
2) In contrast to general belief, dietary carbohydrate plays an important role in regulating drug-metabolizing enzyme activity in the liver; a low-carbohydrate diet enhances, whereas a high-carbohydrate

diet represses, the hepatic metabolism of a variety of volatile hydrocarbons, irrespective of protein and/or fat content(s).

3) Ethanol and carbohydrate are antagonistic in connection with hepatic metabolism: the former increases and the latter decreases it. A decrease (increase) in carbohydrate intake augments (suppresses) the action of ethanol in a dose-related manner: ethanol consumed with lowered carbohydrate intake results in a more remarkable increase in hepatic metabolism than does ethanol consumed with moderate carbohydrate intake.

Alcohol, dietary components, pesticides, and steroids are some of the substances which are discussed further below.

5.4.1. Factors that Increase the Toxicity

The effect of ethanol pretreatment on chloroform-induced heptatoxicity in mice was studied by Kutob and Plaa (1962a). An intoxicating dose (5 g/kg) of ethanol was administered orally to mice daily for 15 days initially, with a systematic shortening of the duration to a single exposure. A challenging dose of chloroform (0.08 ml/kg) was administered subcutaneously either 12, 15, or 24 hours after ethanol treatment. Liver dysfunction was measured by prolongation of phenobarbital sleeping time, bromsulphalein (BSP) retention, liver succinic dehydrogenase activity, and histological examination. Regardless of the ethanol treatment period, phenobarbital sleeping time was significantly increased in mice receiving ethanol followed by chloroform when compared with mice receiving either substance alone. Similar findings were found for BSP retention. The in vitro succinic dehydrogenase activity was significantly reduced by ethanol pretreatment followed by chloroform administration 12 or 24 hours, but not 48 hours, later, when compared with activities from mice receiving only chloroform. Histological changes were seen in the livers of mice given ethanol 15 hours to 4 days prior to chloroform challenge, while mice receiving either chemical alone had morphologically normal livers. It was also determined that the ethanol treatment increased liver triglyceride content, with a maximum at 15 hours, and that ethanol pretreatment significantly increased the concentration of chloroform in the livers with a maximum at 12 hours after chloroform challenge. From these results, it was noted that a single dose of ethanol was just as effective as multiple doses. A mechanism was proposed for the ethanol enhanced chloroform-induced hepatotoxicity in which ethanol increases liver lipid content (as evidenced by increased triglycerides) resulting in increased concentrations of chloroform to be metabolized in the liver.

In support of this mechanism is the observation that oral isopropanol pretreatment for 5 days (0.3 ml/100 g for 2 days and 0.15 ml/100 g for 3 days) followed 12 hours later by 5 daily inhalations of chloroform (5000 ppm first day, 2500 ppm on the next four days), 2 hours/day led to severe fatty infiltration of the liver. Chloroform alone increased the pool of triglycerides (Danni et al., 1981).

In contrast, Sato et al. (1980) studied the mechanism by which ethanol enhances hydrocarbon metabolism, including that of chloroform. Rats ingesting ethanol in their drinking water for 3 weeks were sacrificed 10 hours after the final exposure. Control rats were given isocaloric glucose solutions. Liver microsomal enzyme systems were prepared and liver protein and cytochrome P-450 contents analyzed, the increased contents being indicative of microsomal enzyme synthesis in response to alcohol. When chloroform was added as a substrate, its metabolism was enhanced by 6 times, much more than could be accounted for by enzyme induction alone. Microsomes prepared from rats that were withdrawn from ethanol 24 hours prior to sacrifice did not show enhanced activity. In a subsequent study (Sato et al., 1981), rats receiving a single gavage dose of 0, 2, 3, 4, or 5 g/kg ethanol were sacrificed 18 hours later. The *in vitro* metabolism of chloroform by microsomes prepared from these rats was enhanced very little at 2 g/kg, slightly more at 3 g/kg, and dramatically at 4 g/kg. At 5 g/kg, however, enhanced enzyme activity was no greater than at 3 g/kg ethanol. When ethanol was added directly to the incubation system, the metabolism of chloroform was inhibited. Rats receiving 5 g/kg ethanol retained relatively large amounts in the blood and liver, while those receiving 4 g/kg retained almost none. If the ethanol remaining in the rats exerted an inhibitory effect on enzyme activity, then microsomal enzymes prepared from 5 g/kg ethanol-treated rats, mixed with the soluble fraction from control rats, should show increased activity when compared with both microsomal and soluble fractions from ethanol-treated rats. This was found to be the case.

Based on these results and studies on metabolism of other hydrocarbons *in vitro* and *in vivo*, Sato et al. (1981) suggested that ethanol is both a stimulator and an inhibitor of drug metabolizing enzymes, depending on how much ethanol remains in the body and thus how much time has elapsed since ethanol ingestion. Thus, when ethanol is first ingested, it acts as a competitive inhibitor of microsomal enzyme activity, but as it disappears from the body, an optimum for stimulation may be reached and metabolism enhanced. It was postulated that since the metabolism of chloroform was enhanced to a much greater extent than can be explained from enzyme induction alone, perhaps ethanol modifies the enzyme activities by other mechanisms such as modification of membrane properties, allosteric effects, or by displacement of substrate already bound.

Polybrominated biphenyls (PBBs) have also been found to potentiate the toxicity of chloroform (Kluwe and Hook, 1978). Mice were fed diets containing 0, 1, 25, or 100 ppm PBB for 14 days. One day before sacrifice, the mice were given a single intraperitoneal injection of 0, 0.5, 2.5, 5.0, or 50 µl/kg chloroform. PBB enhanced the toxicity of chloroform in both the liver and the kidney as evidenced by results of blood urea nitrogen (BUN) and serum glutamic oxaloacetic transaminase (SGOT) determinations and by inhibition of p-aminohippuric acid (PAH) uptake by renal slices. PBB also reduced the LD_{50} of chloroform in these mice, and the deaths were attributed to hepatic necrosis. Since PBBs were known to induce the drug metabolizing enzymes, their effects on chloroform were assumed to be due to enhanced chloroform metabolism. Similar findings have been described for other species and other inducers of mixed function oxidases. Thus Abdelsalam et al. (1982) have reported that dieldrin, as does phenobarbital, potentiates chloroform-induced hepatotoxicity of male Nubian goats.

Steroids appear to play a role in the potentiation of chloroform toxicity, especially in the kidney as seen from the sex-related differences in the response of mice (Eschenbrenner and Miller, 1945a; Deringer et al., 1953) and by experiments involving testosterone administration to castrated male mice (Eschenbrenner and Miller, 1945b; Culliford and Hewitt, 1957; Hill, 1978) discussed previously. Clemens et al. (1979) further studied this phenomenon in castrated male and intact female mice. Dose-dependent testosterone sensitization of renal tubules to a fixed dose of chloroform was observed in castrated males with the response ranging from kidney dysfunction to death at high doses. Medroxyprogesterone acetate, an androgenic progestin, enhanced chloroform-induced kidney damage in both castrated males and intact females. Progesterone or hydrocortisone potentiated chloroform toxicity in DBA/2J castrated male mice, but not in the C57BL/J6 strain males nor in any of the females. The mechanism by which the androgens exerted their potentiation may have been mediated through strain specific androgen receptors of the proximal convoluted tubular cells. The mechanism for the potentiating action of the other steroids was less clear.

The potentiation of chloroform toxicity by various ketone and ketogenic substances such as isopropanol, acetone, 2-hexanone and the insecticide chlordecone (Kepone) has been studied extensively in recent years. Hewitt et al. (1979) and Cianflone et al. (1980) have shown that while pretreatment of mice with chlordecone enhanced the liver damage caused by chloroform exposure, the non-ketonic structural analog mirex did not. Animals administered chloroform alone, when compared to those pretreated with chlordecone plus chloroform, have different histological pattern of damage (Hewitt et al., 1979), despite similar rate of chloroform bioactivation (Cianflone et al., 1980). Thus it has been postulated that chlordecone pretreatment may not only bioactivate chloroform metabolism but also perturb the macromolecular localization of the chloroform-derived reactive intermediates, thereby changing the evolution of the toxic process. Hewitt et al. (1983b) compared the pattern of chloroform binding to hepatocyte macromolecular constituents, at various time points, in animals pretreated with chlordecone or its nonpotentiating analog mirex. Macromolecular distribution of chloroform-derived reactive intermediate(s) was assessed by determining irreversibly bound ^{14}C in the protein, lipid and acid soluble fractions after administering ^{14}C-chloroform. Rats and mice were given chlordecone and mirex by gavage 18 hr before ^{14}C-chlorofrom challenge dose and killed 1 hr later. A marked increase of total ^{14}C-hepatic binding occurred with chlordecone but not with mirex. Chlordecone binding was distinguished by a significant increase of protein and lipid binding with a concomitant decrease in acid-soluble fraction binding, i.e., an alteration in binding distribution in hepatocytes. Since mirex is also a known inducer of MFO enzymes (like chlordecone), but lacks the chloroform potentiating ability, these investigators suggest that these two chemicals induce catalytically different forms of cytochrome P_{450} with different substrate specificities (Kaminsky et al., 1978), as well as altering the cellular distribution of chloroform-derived toxic metabolites. Iijima et al. (1983) have noted from morphologic measurements that pretreatment of rats with chlordecone caused a dose-dependent increase in number of cells (area) affected by chloroform (independently of severity), as well as an enhanced severity of cellular change for a given chloroform dose.

Other ketones have also been compared for their ability to enchance the hepatotoxic and nephrotoxic action of chloroform in rats with the following results: methyl n-butyl ketone (MBK) and 2,4-hexanedione were the most potent enhancers, followed by acetone and n-hexane (a ketogenic chemical) (Hewitt et al., 1980). Hewittt et al. (1983a) have noted a positive correlation betweeen ketone carbon chain length (3 to 7C) and the severity of the potential hepatotoxic response to chloroform in rats, even though the ketones at the dose levels given did not themselves produce significant hepatotoxicity. Jernigan and Harbison (1982) speculated that perhaps female mice have greater microsomal enzyme activities, different membrane properties, or perhaps produce a different reactive metabolite of chloroform than do males.

The mechanism of ketone potentiation of chloroform-induced hepato- and nephrotoxicity was also investigated by Branchflower and Pohl (1981) using MBK. Male rats were pretreated with MBK followed by chloroform administration. The metabolism of chloroform by liver and kidney microsomal enzymes and the toxicity to these organs were examined. Control experiments were conducted in which rats were either not pretreated, not given chloroform, or given deuterated chloroform ($CDCl_3$) instead of chloroform. MBK increased cytochrome P-450 levels and NADPH-dependent cytochrome reductase activity in liver microsomes, while having no effect on renal levels of these microsomal components. MBK pretreatment doubled the rate of metabolism of chloroform to diglutathionyl dithiocarbonate (GSCOSG) in microsomal preparations, and more GSCOSG was excreted into the bile of the pretreated animals when compared with rats receiving only chloroform. The amount of GSCOSG in bile was less in MBK-$CDCl_3$ treated animals. GSH levels were significantly decreased by MBK treatment and this decrease was enhanced following chloroform exposure, and to a lesser extent, following $CDCl_3$ exposure. Rats pretreated with MBK followed by chloroform had greatly elevated levels of SGPT associated with liver necrosis and significantly greater BUN levels associated with renal cortical tubule lesions over the control groups. A mechanism was proposed whereby MBK, by increasing cytochrome P-450 levels, enhanced the metabolism of chloroform to phosgene. Furthermore, according to the hypothesis, the phosgene was converted to GSCOSG through GSH, levels of which were diminished by MBK, because the more phosgene formed, the more GSH was depleted in the reaction. The results with $CDCl_3$ indicated that C-H bond was involved in the mechanism. Although MBK also potentiated chloroform toxicity to the kidney, a different mechanism may have been involved since renal cytochrome P-450 and renal GSH levels were not affected.

More recently, Branchflower et al. (1983) have shown that in rat liver MBK potentiates toxicity at least in part by altering the composition of MBK-induced P_{450} isocytochromes that metabolize chloroform to phosgene. Microsomes from MBK and phenobarbital-treated male rats were isolated and subjected to gel electrophoretic and anion-exchange chromatography analysis. The qualitative character of the liver P_{450} cytochromes formed by the two inducers appeared similar. Isolation and reconsititution of the major P_{450} isocytochrome induced by MBK and by phenobarbital showed a parallel ability to form phosgene from chloroform and chloroform-induced hepatotoxicity. However, MBK-induced microsomes were significantly more active than phenobarbital-induced microsomes in the formation of phosgene from chloroform as expressed in terms of nanomoles of GSCOSG formed per nanomole of cytochrome P_{450}. Cowlen et al. (1984) have also investigated the mechanisms of ketone-potentiated toxicity of chloroform. These workers orally dosed rats with 2-hexanone in corn oil and 18 hr later administered a challenge dose of chloroform. The rats were killed 1, 2, and 6 hr later. Hepatic activity of NADPH-succinate-dependent cytochrome C reductases were determined as marker enzymes of endoplasmic reticulum and of mitochondrial membranal function, respectively. Also measured were three indices of lipoperoxidation: formation of conjugated dienes, depletion of unsaturated fatty acids, and production of malondialdehyde. NADPH-dependent cytochrome C reductase activity increased modestly (59%) one hr after chloroform challenge (as compared to chloroform alone), but succinate-dependent cytochrome C activity decreased (87%). Lipoperoxidation was not initiated at the doses of hexanone and chloroform used, although liver damage was increased 30-fold by the ketone as assessed by other measures. These investigators concluded that 2-hexanone potentiation of chloroform hepatotoxicity could not be attributed solely to potentiated lipid peroxidation, but that the changes in succinate-dependent cytochrome C reductase activity suggests the existence of an additional mechanimsm independent of the bioactivation of chloroform.

5.4.2. Factors that Decrease the Toxicity

As discussed above, the experiments of Sato et al. (1980, 1981) indicate that ethanol is both a stimulator and an inhibitor of microsomal enzymes, and hence, of chloroform metabolism and toxicity, depending upon the length of time after ingestion.

Disulfiram and its metabolites have also been studied with respect to their protective effects of chloroform-induced hepatotoxicity (Scholler, 1970; Masuda and Nakayama, 1982). Disulfiram is used in treating chronic alcoholism and is metabolized to carbon disulfide and diethyldithiocarbamate (a herbicide) (IARC, 1976). Disulfiram, a known inhibitor of the microsomal drug metabolizing enzymes, given to rats prior to chloroform anesthesia completely prevented the elevated SGPT activity and liver necrosis observed in rats administered chloroform alone (Scholler et al., 1970). More recently, Masuda and Nakayama (1982, 1983) studied the effects of diethyldithiocarbamate (DTC) and carbon disulfide (CS_2) pretreatment (given 30 min, orally) in male mice challenged with chloroform (i.p., 0.25 ml/kg). DTC (mediated through CS_2 production in stomach) and CS_2 had protective effects on both liver and renal toxicity as measured 24 hr later by SGPT activity, phenolsulfonphalein clearance, liver and kidney calcium accumulation, renal PAH accumulation, and cytopathology. DTC and CS_2 decreased P_{450} content and drug metabolizing enzyme activities in both liver and kidney cortex (but in vitro only in the presence of NADPH), indicating that these compounds must first be metabolized before exerting their inhibitory effect on chloroform metabolism. Gopinath and Ford (1975) also found that DTC or CS_2 protected against chloroform hepatotoxicity in rats, and the effect was presumed to be due to inhibition of drug metabolizing enzymes.

Dietary components can also alter the toxicity of chloroform. It is a widely held opinion that low protein content of the diet decreases microsomal enzyme activities, while a high protein diet increases the activities (McLean and McLean, 1969; Nakajima et al., 1982). If this is the case, a low protein diet should protect against chloroform hepatotoxicity by inhibiting the enzymes responsible for chloroform metabolism. It was found, however, that protein depletion did not alter the toxicity of chloroform in rats given a single oral dose (McLean and McLean, 1969; McLean, 1970). If pretreated with phenobarbital or NDDT to induce microsomal enzymes, rats maintained on a standard diet were no more susceptible to chloroform-induced liver damage than were pretreated, protein-depleted rats (McLean, 1970).

More recently, Nakajima et al. (1982) studied the individual effects of protein, fat, and carbohydrate on the metabolism of chloroform in relation to its toxicity in male rats. Test diets were varied with respect to carbohydrate, protein, or fat while maintaining isocaloric contents. Microsomal enzymes were prepared and chloroform was added as a substrate. The following results were obtained: decreased food intake increased liver microsomal enzyme activities; decreased sucrose content in the diet increased the metabolic rate; varying the protein and fat content, while holding the sucrose content constant, had no effect on the metabolic rate; a carbohydrate-free diet, which contained high protein and high fat, accelerated the rate of chloroform metabolism almost as much as 1 day of food deprivation. The authors concluded that it is a high carbohydrate content, rather than a low protein content, which is responsible for the decreased microsomal enzyme metabolism of chloroform and, hence, its toxicity.

5.5. SUMMARY: CORRELATION OF EXPOSURE AND EFFECT

The purpose of this section is to delineate dose-response relationships for the systemic toxicity of chloroform.

5.5.1. Effects of Acute Inhalation Exposure

The adverse effects on humans of inhaling high concentrations of chloroform have been well documented in the course of its use as an anesthetic. Studies that define the threshold region of exposure for such effects in humans are, however, sparse at best. Experiments involving subchronic exposure of several species of animals give some information on toxicity thresholds for renal and hepatic effects, but little for CNS and none for cardiovascular effects.

The only experimental studies conducted with humans (Lehmann and Hasegawa, 1910; Lehmann and Schmidt-Kehl, 1936) involved relatively short exposures and subjective responses. The results of these studies indicate that the odor of chloroform can be perceived at about 200 ppm. Subjective CNS effects (dizziness, vertigo) apparently did not occur at 390 ppm during a 30-minute exposure but were perceived at about 900 ppm after 2-3 minutes of exposure. Subjects exposed to 1400 ppm for 30 minutes experienced tiredness and headache in addition to the above CNS symptoms. The threshold for "light intoxication" was about 4300 ppm (20 minutes). An exposure duration of 30 minutes or less is insufficient to achieve pulmonary steady state (or total body equilibrium, Section 4.2.3). Hence, longer exposures at these concentrations would be expected to cause more severe effects.

Chloroform concentrations used for the induction of anesthesia ranged from about 20,000-40,000 ppm (NIOSH, 1974; Adriani, 1970) and for the maintenance of anesthesia ranged from 1500 ppm (light anesthesia) to 15,000 ppm (deep anesthesia) (Goodman and Gilman, 1980). Continued exposure to 20,000 ppm could result in respiratory failure, direct depression of the myocardium, and death (Section 5.1.1). Levels of exposure sufficient to produce anesthesia have also caused cardiac arrhythmias and extrasystoles (Kurtz et al., 1936; Orth et al., 1951) and hepatic necrosis and fatty degeneration (Goodman and Gilman, 1980; Wood-Smith and Stewart, 1964).

Data from acute animal exposures tend to show similar CNS effects at roughly the same levels of exposure that produced these effects in humans (Lehmann and Flury, 1943). In addition, some data on the threshold for hepatic effects have been obtained for mice. Kylin et al. (1963), in experiments with female mice of an unspecified strain, found that single, 4-hour exposures to chloroform produced mild hepatic effects (increased incidence of moderate fatty infiltration) at 100 ppm. At 200 ppm, in addition to fatty infiltration, hepatic necrosis and increased serum ornithine carbamyl transferase activity occurred. (An elevation in serum levels of this enzyme indicates liver damage according to Divincenzo and Krasavage, 1974.) Further increases in fatty infiltration, necrosis, and serum enzyme activity were observed at 400 and 800 ppm. These effects appeared to be reversible because the extent of change was less severe 3 days after exposure than it was 1 day after exposure.

Damage to the kidneys of male mice of sensitive strains (e.g., C3H) has occurred at exposure levels as low as 5 mg/L (1025 ppm) for 1 hour (Deringer et al., 1953). The damage consisted of necrosis of the epithelium of the proximal tubules.

5.5.2. Effects of Acute Oral Exposure

Dose-response data for acute oral exposure of humans to chloroform are limited to case reports. A fatal dose of as little as 1/3 ounce (10 ml) was reported (Schroeder, 1965).

A variety of dose-response data is available for acute oral administration of chloroform to animals. Single doses that were sufficient to adversely affect kidney function (measured as excessive loss of glucose and/or protein in the urine in male mice) ranged from 89-149 mg/kg in sensitive and relatively insensitive strains (Hill, 1978). At single oral doses of 1071 mg/kg, but not 756 or 546 mg/kg chloroform, increases in organ weights and mild to moderate lesions were observed in the livers and kidneys of Sprague-Dawley rats (Chu et al., 1982a). In male B6C3F1 mice, renal necrosis occurred after 20 mg/kg, and focal tubular regeneration occurred after 60 or 240 mg/kg but not after 15 mg/kg (Reitz et al., 1980). A low observed adverse-effect level (LOAEL) for hepatic effects in mice can be identified from the study of Jones et al. (1958), in which 30 mg/kg caused midzonal fatty infiltration. Doses in the range of 133-355 mg/kg (Jones et al., 1958; Reitz et al., 1980) represent a FEL (Frank-Effect-Level) for hepatic damage (including centrilobular necrosis) in mice. According to Torkelson et al. (1976), rats given "as little as" 250 mg/kg chloroform "showed adverse effects" on liver and kidney as determined by gross pathological examination. Reported oral LD_{50} values for mice ranged from 119-1400 mg/kg, depending on sex, strain, and age (Kimura et al., 1971; Hill, 1978; Bowman et al., 1978). For rats, LD_{50} values of 908-2000 mg/kg have been reported (Chu et al., 1980; Torkelson et al., 1976). The lethal dose studies included both 24-hour and delayed deaths.

5.5.3. Effects of Dermal Exposure

Chloroform is irritating to the skin. It has been reported to cause degenerative changes in the renal tubules of rabbits exposed dermally to high doses under extreme conditions (1-3.98 g/kg body weight for 24 hours under an impermeable plastic cuff) (Torkelson et al., 1976). In humans, toxicity from dermal exposure is probably not important in comparison with other routes.

5.5.4. Effects of Chronic Inhalation Exposure

Limited information on the effects of long-term intermittent exposure of humans or animals to chloroform is available. A study involving a small number of workers (Challen et al., 1958) indicates that long-term exposure to 20-71 ppm (98-346 mg/m^3) for a 4-8 hour workday, with occasional brief excursions to $\approx$1163 ppm, may represent a LOAEL for symptoms of CNS toxicity. No evidence of liver damage or other organic lesion was detected by physical examination and clinical chemistry tests. A single report linking liver enlargement and viral hepatitis to occupational exposure to 10-200 ppm chloroform (Bomski et al., 1967) is flawed by the apparent lack of suitable controls. The available data do not define a NOAEL (no-observed-adverse-effect-level) or NOEL (no-observed-effect-level) for humans.

Experiments with several species of animals (Torkelson et al., 1976) give some information regarding the threshold region for hepatic and renal effects of inhalation exposure to chloroform (Table 5-3). The animals were exposed to chloroform 5 days/week for 6 months. Exposure to 25 ppm of chloroform for up to 4 hours/day had no adverse effects in male rats as judged by organ and body weights and probably the gross and microscopic appearance of at least the liver and kidneys, although the authors were not explicit about the latter. Exposure to 25 ppm for 7 hours/day, however, produced histopathologic changes in the livers and kidneys of male but not female rats. These changes were characterized as lobular granular degeneration and focal necrosis throughout the liver and cloudy swelling of the kidneys. The hepatic and renal effects appeared to be reversible because rats exposed in the same way, but given a 6-week "recovery period" after the exposure period, appeared normal by the criteria tested. Increasingly pronounced changes were observed in the livers and kidneys of both sexes of rats exposed to 50 or 85 ppm. Hematologic, clinical chemistry, and urinalysis values, tested at the two higher levels of exposure, were "within normal limits."

Similar experiments with guinea pigs and rabbits gave somewhat inconsistent results. Histopathological lesions were observed in liver and kidneys of both species at 25 ppm but not at 50 ppm in either species and not in guinea pigs even at 85 ppm (Torkelson et al., 1976).

The experiments of Torkelson et al. (1976) indicate that subchronic exposure to 25 ppm (123 mg/m^3), 4 hours/day, 5 days/week represents a NOAEL and exposure to 25 ppm, 7 hours/day, 5 days/week respresents a LOAEL for rats. Guinea pigs and rabbits may be slightly less sensitive.

5.5.5. Effects of Chronic Oral Exposure

Few dose-response data for oral exposure of humans to chloroform appear to be available (Chapter 5). A single controlled study has been performed. In this study, subjects were exposed to 70 or 178 mg of chloroform/day ($\approx$1 or 2.5 mg/kg/day assuming 70 kg body weight) for at least 1 year (DeSalva et al., 1975). Neither liver function tests nor blood urea nitrogen determinations (a measure of kidney function) revealed statistically significant differences between exposed and control subjects. Case reports involving abuse of medicines containing chloroform (Wallace, 1950; Conlon, 1963) are not adequate for risk assessment because of the small numbers of patients, exposure to other agents, and imprecise estimates of intake.

Subchronic and chronic toxicity experiments with rats, mice, and dogs, when considered together (Table 5-4), do not clearly establish a NOAEL or NOEL. Although no adverse effects were observed in four strains of mice given 17 mg/kg/day of chloroform, 6 days/week for 2 years (Roe et al., 1979), at the lowest dose level tested (i.e., 15 mg/kg/day, 6 days/week for 7.5 years) in dogs, chloroform treatment was associated with an elevation in SGPT in some but not all of the other tested clinical chemistry indices of hepatic damage (Heywood et al., 1979). The livers of dogs treated with chloroform at this dosage level had larger and more numerous "fatty cysts" than were found in controls. These fatty cysts consisted of aggregations of vacuolated histiocytes. No effect on survival, growth, organ weights, gross and histological appearance of other organs, or hematologic or urinalysis values was observed at this dosage level. Hence 15 mg/kg/day (6 days/week) represents a LOAEL for dogs for effects on the liver. Chronic oral administration of 60 mg/kg/day of chloroform (6 days/week) was associated with slight hepatic changes in rats (Palmer et al., 1979) and with increased incidences of moderate to severe renal disease in male mice of sensitive strains (Roe et al., 1979).

None of the three species tested in long-term experiments appeared to be markedly more sensitive to the toxicity of chloroform than any other; dogs may have been slightly more sensitive. There were considerable differences among strains of mice in the sensitivity of the males to chloroform nephrotoxicity, as had also been observed in acute toxicity experiments.

As would be expected, dosages that produced little or no histologic or clinical chemistry evidence of toxicity when given subchronically (15 and 30 mg/kg/day; rats, dogs) resulted in greater evidence of toxicity when given for longer periods of time (Palmer et al., 1979; Heywood et al., 1979). The response to chloroform in the long-term studies may have been modified by the presence or absence of intercurrent respiratory and renal disease, but no consistent pattern is obvious from an inspection of the data in Table 5-4.

5.5.6. Target Organ Toxicity

Target organs characteristic of the acute toxicity of chloroform are the central nervous system, liver, kidney, and heart. For chronic exposure to chloroform, characteristic target organs are the liver and kidney, and possibly the central nervous system. Some dose-response data are available for the toxicity of chloroform to the liver, kidney, and central nervous system; these data are summarized in Table 5-5 by target organ. The studies from which these data are drawn are discussed more fully elsewhere in Chapter 5, but a comparison on the basis of end point (target organ) was also considered to be useful.

Manifestations of liver damage include centrilobular necrosis, vacuolization, disappearance of glycogen, fatty degeneration and swelling (Groger and Grey, 1979). In the kidney, chloroform exposure produces necrosis of the proximal and distal convoluted tubules (Eschenbrenner and Miller, 1945a). The mechanism by which chloroform produces these effects has been extensively studied in experimental animals. From the studies summarized in Section 5.3.1 (Brown et al., 1974; Ilett et al., 1973; Docks and Krishna, 1976; Ekström et al., 1981; Lavigne and Marchand, 1974; McMartin et al., 1981; Masuda et al., 1980; Harris et al., 1982; Stevens and Anders, 1981), it appears that chloroform is first metabolized in the target organ by microsomal drug metabolizing enzymes to a reactive intermediate, probably phosgene, which in turn can react by various pathways, depending on glutathione levels, one of which is the covalent binding to liver proteins resulting in necrotic lesions. A similar mechanism may or may not occur in the kidney (Kluwe and Hook, 1981).

Table 5-5. TARGET ORGAN TOXICITY OF CHLOROFORM

Target organ	Route and type of exposure	Species	Dose or exposure	Effect on target organ	Reference
liver	inhalation, acute (surgical anesthesia)	human	induction = 20,000-40,000 ppm x a few minutes, plus maintenance = 1500-15,000 ppm x variable duration	necrosis, fatty degeneration in some patients	NIOSH, 1974; Goodman and Gilman, 1980; Wood-Smith and Stewart, 1964
liver	inhalation, acute	mice	100 ppm x 4 hours, single exposure	fatty infiltration	Kylin et al., 1963
liver	inhalation, acute	mice	200 ppm x 4 hours, single exposure	necrosis, fatty infiltration, increase in SOCT	Kylin et al., 1963
liver	inhalation, chronic	rats	25 ppm, 4 hours/day, 5 days/week x 6 months	no effect	Torkelson et al., 1976
liver	inhalation, chronic	rats	25 ppm, 7 hours/day, 5 days/week x 6 months	lobular granular degeneration, focal necrosis	Torkelson et al., 1976
liver	inhalation, chronic	rats	50 or 85 ppm, 7 hours/day, 5 days/week x 6 months	marked centrilobular granular degeneration	Torkelson et al., 1976
liver	oral, acute	mice	30 mg/kg bw; single dose	fatty infiltration	Jones et al., 1958

Table 5-5. (continued)

Target organ	Route and type of exposure	Species	Dose or exposure	Effect on target organ	Reference
liver	oral, acute	mice	133 mg/kg, single dose	massive fatty infiltration and severe necrosis	Jones et al., 1958
liver	oral, acute	mice	355 mg/kg, single dose	massive fatty infiltration and severe necrosis	Jones et al., 1958
liver	oral, acute	mice	60 mg/kg, single dose	no effect	Reitz et al., 1980
liver	oral, acute	mice	240 mg/kg, single dose	hepatocellular necrosis and swelling; inflammation	Reitz et al., 1980
liver	oral (drinking water) subchronic	rats	20, 38, 57, 81, or 160 mg/kg/day x 90 days	transient hepatosis (at 30 and 60, but not at 90 days)	Jorgenson and Rushbrook, 1980
liver	oral (drinking water) subchronic	mice	64 or 97 mg/kg/day x 90 days	transient centrilobular fatty change (at 30 and 60 but not at 90 days)	Jorgenson and Rushbrook, 1980
liver	oral (drinking water) subchronic	mice	145 or 190 mg/kg/day x 90 days	fatty change	Jorgenson and Rushbrook, 1980

Table 5-5. (continued)

Target organ	Route and type of exposure	Species	Dose or exposure	Effect on target organ	Reference
liver	oral (gavage) subchronic	rats	410 mg/kg/day, 6 days/ week x 13 weeks	fatty change and necrosis	Palmer et al., 1979
liver	oral (capsule), subchronic	dogs	30 mg/kg/day, 7 days/ week x 13 weeks	no effect	Heywood et al., 1979
liver	oral (capsule), subchronic	dogs	45 mg/kg/day, 7 days/ week x 13 weeks	slight fatty change	Heywood et al., 1979
liver	oral (capsule), subchronic	dogs	60 mg/kg/day, 7 days/ week x 18 weeks	fatty degeneration, increase in SGOT and SGPT	Heywood et al., 1979
liver	oral (capsule), subchronic	dogs	120 mg/kg/day, 7 days/ week x 12 weeks	fatty degeneration, jaundice, increase in SGOT, SGPT, bilirubin	Heywood et al., 1979
liver	oral (gavage), chronic	rats	60 mg/kg/day, 6 days/ week x 80 weeks	minor histological changes and decrease in relative liver weight	Palmer et al., 1977
liver	oral (gavage), chronic	mice	60 mg/kg/day, 6 days/ week x 80 weeks	no effect	Roe et al., 1979
liver	oral (capsule), chronic	dogs	15 or 30 mg/kg/day, 6 days/week x 7.5 years	increases in SGPT and other serum indicators of hepatic damage, increase in size and number of fatty cysts (vacuolated histiocytes)	Heywood et al., 1979

Table 5-5. (continued)

Target organ	Route and type of exposure	Species	Dose or exposure	Effect on target organ	Reference
liver	oral, chronic	humans	2.5 mg/kg/day for ≥1 year	no effect	De Salva et al., 1975
liver	dermal, acute	rabbits	3.98 g/kg x 24 hours under plastic cuff, single exposure	no macroscopic pathologic changes	Torkelson et al., 1976
kidney	inhalation, acute	mice, males of sensitive strains	5000 ppm, 1 hour, single exposure	necrosis and calcification of tubular epithelium	Deringer et al., 1953
kidney	inhalation, chronic	rats	25, 50, or 85 ppm, 7 hours/day, 5 days/week x 6 months	cloudy swelling of tubular epithelium	Torkelson et al., 1976
kidney	oral, acute	mice, males of sensitive strains	89 mg/kg, single dose	loss of glucose or protein in urine	Hill, 1978
kidney	oral, acute	mice, males of sensitive strains	149 mg/kg, single dose	loss of glucose or protein in urine	Hill, 1978
kidney	oral, acute	mice, male	15 mg/kg, single dose	no effect	Reitz et al., 1980
kidney	oral, acute	mice, male	60 mg/kg, single dose	focal tubular epithelial regeneration	Reitz et al., 1980

Table 5-5. (continued)

Target organ	Route and type of exposure	Species	Dose or exposure	Effect on target organ	Reference
kidney	oral, acute	mice, male	240 mg/kg, single dose	severe diffuse cortical necrosis, focal tubular epithelial regeneration	Reitz et al., 1980
kidney	oral (drinking water) subchronic	rats	160 mg/kg/day x 90 days	no effect	Jorgenson and Rushbrook, 1980
kidney	oral (drinking water) subchronic	rats	≈300 mg/kg/day x 90 days	no effect	Chu et al., 1980b
kidney	oral (drinking water) subchronic	mice	290 mg/kg/day x 90 days	no effect	Jorgenson and Rushbrook, 1980
kidney	oral (capsule), subchronic	dogs	120 mg/kg/day, 7 days/week x 12 weeks	no effect	Jorgenson and Rushbrook, 1980
kidney	oral, chronic	humans	2.5 mg/kg/day, 7 days/week, for ≥1 year	no effect on BUN	De Salva et al., 1975
kidney	oral (gavage), chronic	rats	200 mg/kg/day, 5 days/week x 78 weeks	no effect	NCI, 1976

Table 5-5. (continued)

Target organ	Route and type of exposure	Species	Dose or exposure	Effect on target organ	Reference
kidney	oral (gavage), chronic	mice	138 or 227 mg/kg/day, 5 days/week x 78 weeks	decreased incidence of renal disease	NCI, 1976
kidney	oral (gavage), chronic	mice, males of sensitive strains	17 mg/kg/day, 6 days/week x 80 weeks	no effect	Roe et al., 1979
kidney	oral (gavage) chronic,	mice, males of sensitive strains	60 mg/kg/day, 6 days/week x 80 weeks	increased incidence of moderate to severe renal disease	Roe et al., 1979
kidney	oral (gavage) chronic	mice, males of insensitive strains, females	60 mg/kg/day, 6 days/week x 80 weeks	no effect	Roe et al., 1979
kidney	oral (capsule), chronic	dog	15 mg/kg/day 6 days/week x 7.5 years	no effect	Heywood et al., 1979
kidney	oral (capsule), chronic	dog	30 mg/kg/day, 6 days/week x 7.5 years	increase in fat deposition in glomeruli	Heywood et al., 1979
kidney	dermal, acute	rabbits	1.0, 2.0, and 3.98 g/kg x 24 hours under plastic cuff, single exposure	degenerative changes in tubules	Torkelson et al., 1976

Table 5-5. (continued)

Target organ	Route and type of exposure	Species	Dose or exposure	Effect on target organ	Reference
central nervous system (CNS)	inhalation, acute	humans	900-1400 ppm for ≥30 minutes, single exposure	dizziness, tiredness, headache	Lehmann and Hasegawa, 1910; Lehmann and Schmidt-Kehl, 1936
CNS	inhalation, acute	humans	4300-5100 ppm x 20 minutes, single exposure	dizziness, light intoxication	Lehmann and Hasegawa, 1910; Lehmann and Schmidt-Kehl, 1936
CNS	inhalation, acute	humans	1500-2000 ppm, single exposure	maintenance of light anesthesia (after induction)	Goodman and Gillman, 1980
CNS	inhalation, acute	humans	15,000 ppm, single exposure	maintenance of heavy anesthesia (after induction)	Goodman and Gillman, 1980
CNS	inhalation, acute	humans	20,000-40,000 ppm x a few minutes, single exposure	induction of anesthesia	NIOSH, 1974 Adriani, 1970
CNS	inhalation, acute	mice	2500 ppm x 12 hours, single exposure	no obvious effects	Lehmann and Flury, 1943

Table 5-5. (continued)

Target organ	Route and type of exposure	Species	Dose or exposure	Effect on target organ	Reference
CNS	inhalation, acute	mice	3100 ppm x 1 hour, single exposure	light narcosis	Lehmann and Flury, 1943
CNS	inhalation, acute	mice	4100 ppm x 0.5 hours, single exposure	deep narcosis	Lehmann and Flury, 1943
CNS	inhalation, acute	cats	7200 or 21,500 ppm x 5 minutes, single exposure	disturbance of equilibrium	Lehmann and Flury, 1943
CNS	inhalation, acute	cats	7200 ppm x 60 min single exposure	light narcosis	Lehmann and Flury, 1943
CNS	inhalation, acute	cats	7200 ppm x 93 min single exposure	deep narcosis	Lehmann and Flury, 1943
CNS	inhalation, acute	cats	21,500 ppm x 10 min single exposure	light narcosis	Lehmann and Flury, 1943
CNS	inhalation, acute	cats	21,500 ppm x 13 min single exposure	deep narcosis	Lehmann and Flury, 1943
CNS	inhalation, chronic	humans	20-71 ppm (with excursions to 1163 ppm lasting 1.5-2 min) for 4-8 hours/day, 5 days/week	tiredness	Challen et al., 1958

Table 5-5. (continued)

Target organ	Route and type of exposure	Species	Dose or exposure	Effect on target organ	Reference
CNS	inhalation, chronic	humans	77 to 237 ppm (with excursions to ≈1163 ppm lasting 1.5-2 min) for 4-8 hours/day, 5 days/week	tiredness, depression, occasional silliness or staggering during the workday	Challen et al., 1958
CNS	oral, acute	rats	350 mg/kg single dose	minimum narcotic dose (MND_{50})	Jones et al., 1958
CNS	oral (drinking water), subchronic	rats	20-160 mg/kg/day x 90 days	dose-related signs of depression during 1st week only	Jorgenson and Rushbrook, 1980
CNS	oral (drinking water), subchronic	mice	32-290 mg/kg/day x 90 days	dose-related signs of depression during 1st week only	Jorgenson and Rushbrook, 1980
CNS	oral (drinking water) subchronic	rats	≈300 mg/kg/day x 90 days	no histopathologic changes in brain	Chu et al., 1982b
CNS	oral (gavage), subchronic	rats	60 mg/kg/day 6 days/week x 80 weeks	no effect on gross or histologic appearance of brain	Palmer et al., 1977

5.5.7. Factors that Modify the Toxicity of Chloroform

Several substances alter the toxicity of chloroform, most probably by modifying the metabolism of chloroform to a reactive intermediate (see Section 5.4). These substances are of interest because humans may be accidentally or intentionally exposed to them. Factors that potentiate the toxic effects induced by exposure to chloroform include ethanol (Kutob and Plaa, 1962; Sato et al., 1980, 1981), polybrominated biphenyls (Kluwe and Hook, 1978), steroids (Clemens et al., 1979), and ketones (Hewitt et al., 1979; Jernigan and Harbison, 1982; Branchflower and Pohl, 1981). Disulfiram and its metabolites (Scholler et al., 1970; Masuda and Nakayama, 1982; Gopinath and Ford, 1975) and high carbohydrate diets (Nakajima et al., 1982), appear to protect against chloroform toxicity.

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6. TERATOGENICITY AND REPRODUCTIVE EFFECTS

Schwetz et al. (1974) evaluated the effects of reagent grade chloroform (lot no. 9649, Burdick and Jackson Laboratories, 99.3% chloroform, 0.65% carbon tetrachloride, and 0.03% unknown) on the maternal and fetal well-being of Sprague-Dawley rats. Twenty female rats were exposed, by inhalation (7 hours/day) to 30, 100, or 300 ppm chloroform on days 6-15 of gestation. The authors analyzed the results statistically using Fisher's Exact Probability Test, analysis of variance, Dunnett's test, or Tukey's test to compare the frequency of anomalies, resorptions, maternal and fetal weights, body lengths, liver weights, or serum glutamic pyruvic transaminase (SGPT) activity in the exposed versus the control groups. The level of significance was chosen at $p < 0.05$, and the litter was used as the experimental unit.

Animals exposed to the highest dose of chloroform (300 ppm) had significant increases in the number of resorptions and a decrease in the apparent conception rate (Schwetz et al., 1974). At the lower doses (30 and 100 ppm), no alterations in resorption rate, fetal body weight, conception rate, number of implantations, or average litter size were observed. Fetal crown-rump length was significantly decreased at 30 and 300 ppm, but not at the 100 ppm level. At 100 ppm, increases in the incidence of acaudia (absence of tail), short tail, imperforate anus, subcutaneous edema, missing ribs, and delayed ossification of sternebrae were observed. At 300 ppm, subcutaneous edema and abnormalities of the skull and sternum were observed, but the incidence of these was not statistically significant. The authors pointed out that small numbers of survivors in the 300 ppm group (4±7 versus the control 10±4 live fetuses/litter) may have prevented adequate statistical evaluation of this effect.

In this study (Schwetz et al., 1974), chloroform produced evidence of maternal toxicity, such as a decrease in the rate of maternal weight gain at all dose levels and a decrease in food consumption during pregnancy at the 100 and 300 ppm level (Table 6-1). Other maternal effects include changes in liver weight gain during pregnancy (no change in absolute liver weight gain at 30 ppm, but an increase at 100 ppm, possibly due to the concomitant anorexia at this dose). No significant changes in SGPT activity were observed in groups exposed to chloroform at the 100 and 300 ppm levels. Developmental effects observed in the 300 ppm group were associated with anorexic effects in the mother, and to control for this effect a starvation group was included in this study. The starvation control group was restricted to a level of food consumption comparable to the 300 ppm chloroform group. Animals on starvation diets (allowed 3.7 g/day of food on days 6-15) had a significant decrease in the absolute weight of the liver and an increase in the relative weight of the liver (see Table 6-1). The effects of 300 ppm chloroform on the increase in the relative weight of the liver were much greater than those of starvation alone. Additionally, exposure to 300 ppm chloroform resulted in a dramatic decrease in the number of animals pregnant at sacrifice (15% pregnant versus 88% in air control), a decrease in the number of live fetuses per litter (4 versus 10 live fetuses/litter), and an increased percentage of litters with resorptions (100% versus 57%). Examination of the uteri indicated that the conceptus had been completely resorbed very soon after implantation. These effects appeared to result primarily from chloroform exposure, and not from influences of maternal stress, since anorexia and liver weight changes associated with starvation were not accompanied by embryotoxic or teratogenic effects (see Table 6-1).

TABLE 6-1. SUMMARY OF RESULTS OF THE SCHWETZ et al. (1974) STUDY

Chemical: Chloroform, reagent grade, lot no. 9649, Burdick and Jackson Laboratories
Animal: Sprague-Dawley rats, 20 animals per group
Route of exposure: Inhalation, 30, 100, 300 ppm
Duration of exposure: 7 hours/day, days 6-15 of gestation

Summary of developmental effects

Chloroform concentration, (ppm)	Air control	Air control (starved)	30	100	300
% pregnancy	88(68/77)	100(8/8)	71(22/31)	82(23/28)	15(3/20)[a]
live fetuses/ litter	10±4	10±4	12±2	11±2	4±7[a]
% resorptions/ implantation	8(63/769)	7(6/87)	8(24/291)	6(16/278)	61(20/33)[a]
% litters with resorptions	57(39/68)	25(2/8)	68(15/22)	52(12/23)	100(3/3)
fetal body weight, g	5.69±0.36	5.19±0.29	5.55±0.20	5.59±0.24	3.42±0.02[a]
fetal crown-rump length, mm	43.5±1.1	42.1±1.1	42.5±0.6	43.6±0.7	36.9±0.2[a]

[a] Significantly different from controls.

SOURCE: Schwetz et al., 1974.

(continued on next page)

TABLE 6-1. (continued)

Summary of maternal effects

Chloroform concentration		Air control	Air control (starved)	30	100	300
Absolute liver weight (g)		36±3	42±4	35±4	41±3	53±11
body weight gain during gestation	gestation day					
	6	275±21	274±13	266±14	274±17	284±+9
	13	310±17	223±13[a]	280±14[a]	274±18[a]	192±9[a]
	21	389±28	326±24[a]	381±23	365±22	241±29[a]
maternal food consumption	gestation day					
	4-5	19±4	19±2	20±3	20±3	18±1
	6-7	19±3	starved	3±3	13±4[a]	1+±1[a]
	8-9	20±3	starved	18±1	15±2[a]	4±2
	10-11	23±2	starved	18±2	16±2[a]	1±1
	12-13	22±2	starved	20±1	15±2[a]	1±1[a]
	14-15	23±3	starved	21±2	19±2[a]	1±1[a]
	16-17	25±4	21±2[a]	27±3[a]	30±3[a]	12±2[a]
	18-19	26±3	24±8[a]	29±5	33±3[a]	----

[a] Significantly different from controls.

SOURCE: Schwetz et al., 1974.

Murray et al. (1979) evaluated the effects of chloroform (spectral grade, Mallinckrodt, lot CSZ, code 4434, purity not reported) administered by inhalation (7 hours/day) in the mouse. Thirty-five animals per group were exposed on gestation days 1-7 or 6-15; forty animals per group were exposed on gestation days 8-15. Only one dose, 100 ppm, was tested during the three different time periods (days 1-7, 6-15, or 8-15 of gestation) in CF-1 mice. The various exposure periods were designed to evaluate the effects of chloroform in very early pregnancy, organogenesis, and somewhat later in pregnancy. Sodium sulfide staining of the uteri was used to detect resorptions in very early pregnancies.

The authors (Murray et al., 1979) analyzed the results statistically using the Fisher's Exact Probability Test to evaluate pregnancy incidence; the modified Wilcoxan test for fetal outcomes; the Mann-Whitney signed rank test for SGPT activity; and one-way analysis of variance for fetal body weights and body measurements, maternal body weights, liver weights, food consumption, and number of implantations and resorptions. The authors chose the level of statistical significance at $p < 0.05$.

Murray et al. (1979) reported that 100 ppm chloroform significantly reduced the ability of the mice to maintain pregnancies when the animals were exposed on days 1-7 or 6-15 of gestation but not on days 8-15 (see Table 6-2 for summary of data). No significant effect was reported on the average number of implantation sites. In animals exposed on days 1-7 of gestation, but not in those exposed on days 6-15 or 8-15, there were significant increases in resorptions per litter. This effect was accounted for by the loss of two entire litters. Mean fetal body weight and crown-rump length were significantly decreased in the groups exposed on days 1-7 and 8-15, but not in those exposed on days 6-15. Maternal toxicity (slight decrease in

TABLE 6-2. SUMMARY OF RESULTS OF THE MURRAY et al. (1979) STUDY

Chemical: Chloroform, spectral grade, Lot CSZ, Code 4434, Mallinckrodt
Animal: CF-1 mice, 35 animals in groups exposed on days 1-7 and 6-15; 40 animals in groups exposed on days 8-15
Route of exposure: Inhalation, 100 ppm (one dose only)
Duration of exposure: 7 hours/day, days 6-15 of gestation

Days	Number Pregnant		Additional pregnancies (sodium sulfide stain)		Total pregnancies	
	Control	Exposed	Control	Exposed	Control	Exposed
1-7	22/35(63%)	11/34(32%)	4	4	26/35(74%)	15/34(44%)
6-15	29/34(85%)	13/35(37%)	2	2	31/34(91%)	15/35(43%)
8-15	25/40(62%)	18/40(45%)	1	6	26/40(65%)	24/40(60%)

	Days of gestation		
	1-7	6-15	8-15
Fetal effects	resorption[a]	---	---
	fetal body weight and crown-rump length[b]	---	fetal body weight and crown-rump length[b]
	---	---	cleft palate[a]
	delayed skeletal ossification[a]	---	delayed skeletal ossification[a]
Maternal effects	body weight gain[b]	body weight gain[b]	body weight gain[b]
	---	liver weight[a]	liver weight[a]
	---	SGPT (only one dose)[a]	---

[a]Significantly increased.
[b]Significantly decreased.

SOURCE: Murray et al., 1979.

body weight gain during pregnancy) was seen in groups exposed on days 6-15, with a more severe decrease in groups exposed on days 1-7 and 8-15. Less food and water were consumed in all experimental groups as compared to controls. Absolute and relative weight of the liver were increased in groups exposed on days 6-15 and 8-15, but not in those exposed on days 1-7. SGPT activity was increased in mice exposed on days 6-15, which was the only time period evaluated for this measurement.

A summary of this study (Murray et al., 1979) is presented in Table 6-2. Chloroform caused a decrease in pregnancy maintenance. The authors concluded that chloroform affected the stages either prior to, or during, early implantation. However, because of the small numbers of animals and the lack of dose-response evaluation (only one dose was tested, 100 ppm), this conclusion must be considered tentative. Other results of the Murray et al. (1979) study indicated that the incidence of cleft palates increased in pups exposed in utero on days 8-15 of gestation, but not on days 1-7 or 6-15. The authors suggested three possibilities to explain this result. The first was that earlier exposure on days 6-15 prevented susceptible offspring from implanting. The second possibility was that the number of litters available (11 in the group exposed on days 6-15) was insufficient to detect this effect. The third was that the teratogenic effect (cleft palate) did not occur in offspring exposed on days 1-7 since they were exposed before organogenesis. The number of offspring coming to term was consistently less in all exposure groups than in the controls (days 1-7, 9 litters versus 22 in control; days 6-15, 11 litters versus 29 in control; days 8-15, 18 litters versus 24 in control). Since the pups with cleft palate were also retarded in growth, the authors suggested that the ability of chloroform to cause malformations was indirect embryotoxic effect and not direct teratogenic effect. However, this speculation was not supported by experimental results and does not alter the conclusion that chloroform is a potential developmental toxicant.

In summary, this study (Murray et al., 1979) indicated that chloroform administration (100 ppm) by inhalation (7 hour/day) produced teratogenic and embryotoxic effects, interfered with pregnancy, and caused maternally toxic effects (changes in liver weight and decreases in weight gain during pregnancy). Exposure in the early stages of pregnancy appeared to produce a decreased incidence of conception, but the results of this study did not conclusively determine which days of pregnancy were most susceptible to the effects of chloroform. To answer this question, it would be necessary to use a greater number of doses and larger numbers of animals per dosage group.

Thompson et al. (1974) investigated the effect of chloroform administered orally, using Sprague-Dawley rats and Dutch-Belted rabbits. The rats were intubated with chloroform (Mallinckrodt, Batch ZJL dissolved in corn oil, purity not reported) twice a day in divided doses of 20 to 516 mg/kg/day. The rabbits were intubated once a day in doses of 20 to 398 mg/kg/day. Each study was divided into two parts, a range-finding portion designed to establish the proper dose range (six rats were administered 79, 126, 300, 316, or 516 mg/kg/day of chloroform; five rabbits were administered 63, 100, 159, 251, or 398 mg/kg/day), and a teratologic study, using greater numbers of animals and three doses (25 rats were administered 20, 50, or 126 mg/kg/day and 15 rabbits were administered 20, 30, and 50 mg/kg/day). The rats were exposed to chloroform on days 6-15 of gestation, while the rabbits were exposed on days 6-18 of gestation.

Statistical evaluations of maternal body weight gains, food consumption, implantations, corpus luteum, resorptions, litter size, and fetal weights were made by an analysis of variance and Dunnett's Two-Tailed Multiple Range Test. Sex ratios and frequency of anomalies among the fetal population and among litters were analyzed by the chi-square test. In all analyses, the level of significance chosen was $p < 0.05$.

The data for the range-finding portion of this study (Thompson et al., 1974) were not presented; however, the authors reported that rats treated orally with greater than 126 mg/kg/day chloroform had signs of maternal toxicity, such as a decrease in food consumption, acute toxic nephrosis, hepatitis, and gastric erosion. Fetal development was adversely affected in rats receiving 316 and 501 mg/kg/day with an increase in the number of resorptions and a decrease in fetal viability, litter size, and fetal weight in dams given 316 mg/kg/day. Only two rats survived when given 501 mg/kg/day; one was not pregnant, the other had complete early resorptions. In a teratologic study (Table 6-3), rats receiving 50 and 126 mg/kg/day displayed signs of maternal toxicity (lowered body weight gain, lowered food consumption, and fatty changes in the liver). No overt toxic effects were observed in animals given 20 mg/kg/day, and no malformation was noted at any dose level.

In the range-finding portion of the study by Thompson et al. (1974) using rabbits, there were signs of maternal toxicity such as severe acute hepatitis and nephrosis, which were observed in animals given 63 mg/kg/day and higher. No overt signs of toxicity were observed at the 25 mg/kg/day level. In the two surviving dams given 100 mg/kg/day, one had four resorption sites with no viable offspring, while the other was not pregnant. No other embryotoxic or teratogenic effect was observed. In the teratologic study of rabbits (Table 6-3), maternal toxicity (depressed weight gain) was observed at the 50 mg/kg/day level. In the fetus, mean body weight was

TABLE 6-3. SUMMARY OF EFFECTS OF THE THOMPSON et al. (1974) STUDY

Chemical: Chloroform NF, Mallinckrodt Batch ZJL
Animal: Sprague-Dawley rats, 25 animals per group, Dutch-Belted rabbits, 15 animals per group
Route of exposure: Rats intubated with 0, 20, 50, 126 mg/kg/day
rabbits intubated with 0, 20, 35, 50 mg/kg day
Duration of exposure: Rats, 6-15 of gestation, rabbits 6-18 of gestation

Maternal effects, teratologic study

rats	20 mg/kg/day	50 mg/kg/day	126 mg/kg/day
rabbits	20 mg/kg/day	35 mg/kg/day	50 mg/kg/day
rats	no overt toxic effect	body weight[a]	food consumption[a]
rabbits		body weight[a]	

Fetal effects, teratology study

rats	20 mg/kg/day	50 mg/kg/day	126 mg/kg/day
rabbits	20 mg/kg/day	35 mg/kg/day	50 mg/kg/day
rats	no significant effect	no significant effect	no significant effect
rabbits	mean body weight[a]		mean body weight[a]

[a] significantly decreased.

SOURCE: Thompson et al., 1974.

depressed at the 20 and 50 but not 35 mg/kg/day levels. No treatment-related effect or major abnormalities were observed although there was an increase in the numbers of offspring with incomplete skeletal when analyzed statistically among all fetuses, but not among litters at the 25 or 35 mg/kg/day level.

In this study (Thompson et al., 1974), adverse effects such as skeletal deformities and deficiencies in pregnancy maintenance reported by Schwetz et al. (1974) and Murray et al. (1979), were not observed. Fetotoxicity in the form of reduced birth weights was observed at the highest doses which were also toxic to the dam. The authors suggested that the greater maternal toxicity observed in this study compared to that of others (Schwetz et al., 1974; Murray et al., 1979) might be attributable to differences in the route or duration of exposure. In the study by Thompson et al. (1974), rats and rabbits were exposed orally to chloroform once or twice a day, while Schwetz et al. (1974) and Murray et al. (1979) administered chloroform by inhalation 7 hour/day. No data are available on the influence of different routes of exposures.

Burkhalter and Balster (1979) evaluated the potential of chloroform to adversely affect behavior in developing ICR mice. This study was designed as a preliminary screening study, with the parental generation of male and female mice (five animals) exposed 21 days prior to mating, during mating, and for an additional 21 days. The offspring were exposed starting on day 7 until day 21 after birth. Only one oral dose of chloroform, 31.1 mg/kg/day, was administered by gavage to parental generation and offspring. Each litter was reduced to eight pups, and three pups were randomly selected for behavioral teratogenic testing. The chloroform (Mallinckrodt, nanograde purity) was administered by gavage and delivered in a solution of 1 part polyoxyethylated vegetable oil, Emulphor (EL-620, GAF Corp., New York), and 8 parts saline. A variety of behavioral responses were evaluated which included righting reflex, forelimb placing response, forepaw grasp, rooting reflex, cliff drop aversion, auditory startled response, bar-holding ability, eye opening, motor performance, and learning ability. The scoring for these responses was based upon predesignated criteria. These criteria established behavioral ability by measuring both objective standards (time to complete test) and subjective standards ("weak" or "complete" grasp of paws).

Body weight and latency to enter the dark compartment in the passive avoidance test were analyzed statistically by repeated measures of the analysis of variance. Screen test latencies were analyzed by t-test. The data from the neurobehavioral developmental scale were analyzed using a Mann-Whitney U test. The level of statistical significance was chosen at $p < 0.05$.

Burkhalter and Balster (1979) reported that the sizes of litters were similar for both the control and experimental groups; however, fetal body weight gain of pups during the 14 days of exposure (days 7-21 following birth) was decreased. Forelimb placement response was reduced in the exposed group on day 5 and 7 of birth, but not on day 9. The significance of this reduction is not known, although the recovery on day 9 suggested that the effect was not indicative of serious delays in behavioral response. The other behavioral responses were not significantly different in the exposed groups. Burkhalter and Balster (1979) concluded that 31.1 mg/kg/day of chloroform produced no significant adverse behavioral effects in pups exposed both _in_ _utero_ and after birth (days 7-21). However, since this study was designed as a preliminary screening study, using one dose and small numbers of animals, there was no attempt to evaluate the full range of dose-related effects. In future studies, it would be desirable to evaluate at least three doses, including doses high enough to produce some maternal toxicity, in addition to using a larger sample size.

In an abstract, Dilley et al. (1977) reported the effects of exposure to chloroform on pregnant rats (strain not reported, number of animals not reported). The animals were exposed by inhalation (20 ± 1.2 g/m^3/day) on days 7-14 of gestation. Two lower concentrations were administered in the study, but the doses were not reported in the abstract. Dilly reported that chloroform increased fetal mortality and decreased fetal weight gain; however, there were no malformations.

In another abstract, Ruddick et al. (1980) reported the results of a study using 15 Sprague-Dawley rats administered 100, 200, and 400 mg/kg/day of chloroform by gavage on days 6-15 of gestation. Chloroform (purity not reported) was reported to cause maternal toxicity (changes in weight gain, biochemical and hematological parameters, and liver or kidney changes). Chloroform also produced adverse effects in fetal development (type not specified), but the authors attributed these effects primarily to maternal toxicity and not directly to chloroform exposure. Without the presentation of this data, however it is not possible to fully evaluate these results.

6.1 SUMMARY

In summary, the results of four articles and two abstracts indicated that under the conditions of the experiments, chloroform has the potential for causing adverse effects in pregnancy maintenance, delays in fetal development, and production of terata in laboratory animals. The adverse effects on the conceptus were observed in association with maternal toxicity; however, the type and severity of effects appeared to be specific to the conceptus, affecting the fetus to a much greater degree than the mother (Schwetz et al., 1974; Murray et al., 1979). The results of a preliminary screening study indicated that a single dose of 31.1 mg/kg/day chloroform has no significant effect on neonatal behavior (Burkhalter and Balster, 1979). Thompson et al. (1974) reported that chloroform does not cause adverse fetal effects except at maternally toxic levels. The two abstracts did not contain enough detail for critical scientific review (Dilley et al., 1977; Ruddick et al., 1980).

The studies in which chloroform was administered by inhalation for 7 hours/day (Schwetz et al., 1974; Murray et al., 1974) reported more severe outcomes than other studies which administered chloroform by intubation once or twice a day. However, since the pharmacokinetic relationship associated with route or duration of exposure has not been studied, it is not possible to evaluate the importance of the route of exposure in causing adverse reproductive outcome. To evaluate more fully the influence of these factors, additional investigations would have to be conducted.

6.2 REFERENCES FOR CHAPTER 6

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7. MUTAGENICITY

7.1. INTRODUCTION

The mutagenic potential of chloroform has been assessed on the basis of several in vitro bacterial studies, one host-mediated assay with Salmonella as the indicator organism, one yeast study, one Drosophila sex-linked recessive lethal test, one mammalian cell culture mutagenicity assay, two sperm head abnormality tests, four cytogenetic studies, and seven DNA damage studies (sister chromatid exchange and unscheduled DNA synthesis). Several assays from a recently published screening study, in which 42 chemicals were tested in various short-term protocols, are also briefly discussed. Information on the binding of metabolically activated chloroform to cellular macromolecules is presented before the sections on genetic damage. Most of the mutagenicity tests have been negative, but there is some evidence that chloroform may be mutagenic. Suggestions are made for further testing.

7.2. COVALENT BINDING TO MACROMOLECULES

As mentioned in Chapter 4, the primary reactive metabolite of chloroform is phosgene, $COCl_2$. The toxicity and carcinogenicity of chloroform may be related to its metabolism to phosgene, which is a crosslinking agent and may bind to macromolecules covalently and crosslink them. Several studies on the binding potential of metabolically activated chloroform have been described in Chapter 4, section 4.6. Other studies and additional information on studies summarized in Chapter 4 are covered below.

Diaz Gomez and Castro (1980a) assessed the potential of purified rat liver nuclei to activate chloroform by measuring covalent binding to nuclear protein and lipid. The results were compared to results obtained from similar incubation mixtures containing microsomes instead of purified nuclei.

The mixtures containing either nuclei (1.3 mg protein/ml) or microsomes (1.56 mg protein/ml) were incubated for 30 min in 6.4 nM $^{14}CHCl_3$ (5.4 Ci/mol) and an NADPH generating system. The extent of binding to protein in the nuclear preparation was approximately 40% of that in the microsomal preparation (nuclei, 27 pmol/mg; microsomes, 68 pmol/mg). Thus, isolated nuclei were less efficient than microsomes in metabolizing chloroform, but the results were within the same order of magnitude.

This study suggests that metabolism of chloroform to a reactive intermediate(s) can occur in association with nuclear membranes, as may be the case with other xenobiotics (Weisburger and Williams, 1982). However, contamination of the nuclear preparations with trace amounts of endoplasmic reticulum may have been sufficient to explain at least part of the nuclear activation observed.

In a subsequent study, Diaz Gomez and Castro (1980b) exposed mouse liver DNA or RNA to $^{14}CHCl_3$ *in vivo* or *in vitro*. For the *in vivo* experiments, the animals were pretreated with phenobarbital or 3-methylcholanthrene and exposed to $^{14}CHCl_3$ by intraperitoneal injection either once daily for 4 days or twice weekly for 2 weeks. The specific activity of the $^{14}CHCl_3$ sample injected was 13.15 μCi/mmol (10% in olive oil) administered at 5 mg/kg. The animals were killed 6 hr after the last injection and the livers were analyzed for binding of $^{14}CHCl_3$ to DNA and RNA. For the *in vitro* study, the investigators added 2.5 x 10^5 dpm $^{14}CHCl_3$, microsomes (9 mg of microsomal protein), cofactors, and 4 mg of mouse liver DNA to 3 ml of reaction mixture. The reaction mixtures were incubated for 30 min at 37°C. It is not clearly stated whether microsomes from chemically pretreated animals were used in the *in vitro* study. The background counts for both the *in vivo* and *in vitro* experiments were 150-160 dpm. The authors observed no significant binding of ^{14}C to the nucleic acids in both the in vivo and in vitro experiments.

DiRenzo et al. (1982) studied in vitro covalent binding of chloroform to calf thymus DNA following bioactivation by liver microsomes isolated from phenobarbital-treated rats. $^{14}CHCl_3$ (5.4 Ci/mol, New England Nuclear) was mixed with unlabeled chloroform and diluted in ethanol so that 1 μmol contained 2-4 x 10^5 dpm (A.J. Gandolfi, personal communication). The $^{14}CHCl_3$ (2 μmol or 4-8 x 10^5 dpm) was incubated with 2 mg of microsomal protein, 5 mg of calf thymus DNA, and an NADPH-generating system in 2.5 ml of 0.05 M Tris-HCl, pH 7.4. After 60 min, the reaction was stopped with cold ethanol and DNA was isolated by pronase digestion followed by extraction with chloroform/isoamyl alcohol (24:1) and precipitation with cold ethanol/sodium chloride. Background was 40-50 dpm (A.J. Gandolfi, personal communication). Protein as analyzed by the Coomassie technique was < 10 μg/mg of DNA. Chloroform bound to DNA at 0.46 ± 0.13 nmol/mg DNA/hr. The majority of the radiolabel cosedimented with native DNA and putative partially hydrolized DNA in CsCl gradients.

When the two in vitro binding studies (Diaz Gomez and Castro, 1980b; DiRenzo et al., 1982) are compared, it is clear that DiRenzo and coworkers optimized binding conditions, which may be the reason they obtained positive results. DiRenzo et al. (1982) added 2- to 3-fold more dpm to the reaction mixtures, incubated the mixtures twice as long, and had about 25% of the background levels, when compared with Diaz Gomez and Castro (1980b). In addition, DiRenzo et al. (1982) used microsomes from chemically pretreated animals, whereas it was not clearly stated whether the microsomes Diaz Gomez and Castro (1980b) used in their in vitro studies were from chemically pretreated animals. The signifiance of in vitro binding for in vivo exposure is unclear. If glutathione were present in the reaction mixtures to more nearly simulate in vivo conditions, DiRenzo and coworkers may not have observed binding. Also, binding at the low levels observed could have been due to a reactive impurity in the radiochemical sample.

In summary, binding of metabolically activated chloroform to liver microsomal and nuclear protein and lipid has been observed. The only conclusion that can be made from the available studies on the DNA binding potential of metabolically activated chloroform is that, if DNA binding does occur, it is probably at a very low level.

7.3. MUTAGENICITY STUDIES IN BACTERIAL TEST SYSTEMS

Uehleke et al. (1977) tested chloroform for mutagenicity in suspension assays with S. typhimurium strains TA1535 and TA1538. No mutagenic activity was observed. About 6-9 x 10^8 bacteria were incubated for 60 min under N_2 in tightly closed test tubes with 5 mM chloroform and microsomes (5 mg protein) plus an NADPH generating system. The mutation frequencies (His^+ colony forming units/10^8 his^- colony forming units) were less than 10 for both strains and the spontaneous mutation frequencies were 3.9 ± 3.7 for strain TA1535 and 4.4 ± 3.5 for strain TA1538. At this concentration of chloroform, survival of the bacteria was at least 90%. Higher concentrations should also have been tested, because mutagenicity is sometimes observed only at higher toxicities. Dimethylnitrosamine (50 mM), cyclophosphamide (0.5 mM), 3-methylcholanthrene (0.1 mM), and benzo(a)pyrene (0.1 mM) were the positive controls.

Studies demonstrating that metabolically activated chloroform binds to protein and lipid in the presence of rabbit microsomes were also described in the paper by Uehleke et al. (1977) and are mentioned in Chapter 4 of this document. However, it is not clear whether rat, mouse, or rabbit microsomes were used in the mutagenicity studies in this paper. If mouse or rat microsomes were used for the mutagenicity tests, it cannot be assumed that chloroform was sufficiently activated, because only rabbit microsomes activated $^{14}CHCl_3$ sufficiently for binding to macromolecules in this study. Another deficiency in this study is that Salmonella strains TA98 and TA100 were not used; these strains contain the R factor plasmid pKM101, which increases the sensitivity of the tester strains to certain mutagens.

The mutagenicity of chloroform was tested in a study on the mutagenic potential of 71 chemicals identified in drinking water (Simmon et al., 1977). Chloroform was tested at 10% by volume (1.24 M) in a suspension assay with Salmonella strains TA1535, TA1537, TA1538, TA98, and TA100; this concentration of chloroform exceeds its solubility. Metabolic activation was provided by S9 mix prepared from Aroclor 1254-treated rats. Mutagenic activity was not observed, but no information on toxicity was provided.

Simmon et al. (1977) also tested chloroform in a desiccator to assess mutagenicity due to vapor exposure. Agar plates were placed uncovered in a desiccator above a glass petri dish containing the chloroform. The desiccator contained a magnetic stirrer that acted as a fan to promote evaporation of the measured amount of chloroform and to maintain an even distribution of the vapors. Plates were exposed to the vapors for 7-10 hr and then removed from the desiccators, covered, and incubated approximately 40 hr before scoring. As in the suspension assay, mutagenic activity was not observed and no information on toxicity was provided.

The study of Simmon et al. (1977), although lacking in details of the chloroform assay, clearly identifies other trihalomethanes ($CHBr_3$, $CHBr_2Cl$, $CHBrCl_2$) as mutagens in the vapor assay in desiccators. Methyl bromide, methyl chloride, methyl iodide, and methylene chloride were also found to be mutagenic in the desiccator assay. However, these seven halogenated compounds did not require metabolic activation to exhibit mutagenic activity.

Kirkland et al. (1981) studied the mutagenicity of chloroform in Escherichia coli strains WP2p and WP2uvrA$^-$p, using reversion to tryptophan prototrophy as the end point. The bacteria were treated with chloroform in plate incorporation and preincubation tests both with and without rat liver microsomes (plus cofactors) prepared from Aroclor 1254-induced rats. The concentration of protein in the microsomal suspension was not given. Chloroform was added at 10,000, 1,000, 100, 10, 1, or 0.1 µg/plate. Negative results were obtained in both tests. In the plate incorporation test, the procedure used to prevent loss of chloroform may have been inadequate; chloroform was added to bacterial suspensions in molten agar, and each mixture was mixed rapidly on a Whirlimixer and poured onto agar plates. Loss of chloroform could have occurred during this mixing procedure. The plates were incubated in gas-tight containers, but it is unlikely that this would prevent evaporation of chloroform from the plates. 2-Aminoanthracene was the positive control requiring metabolic activation, and N-methyl-N'-nitro-N-nitrosoguanidine was the direct-acting positive control.

In a subsequent study from the same laboratory (Van Abbe et al., 1982), two experiments were carried out: the standard plate procedure in which no precautions were taken to control evaporation of chloroform and a vapor-phase experiment in which the bacteria were exposed to chloroform vapors for 2-8 hr. Because chloroform is associated with the development of liver tumors in mice and kidney tumors in rats (NCI bioassay, 1976) the standard plate assay, which usually employs only rat liver S9 mix when activation is desired, included Aroclor-induced S9 mixes from mouse and rat livers and kidneys. Strains TA1535, TA1537, TA1538, TA98, and TA100 and chloroform doses of 0-10 mg/90-mm plate were tested in the standard plate incorporation assay. Negative results were obtained at all doses, both in the absence of metabolic activation or in the presence of S9 mixes from all sources. Toxicity (incomplete bacterial lawn) was observed at 10 mg/plate. All of the positive controls tested were mutagenic under similar conditions.

In the vapor phase study (Van Abbe et al., 1982), the protocol differed from the standard procedure. The bacterial suspension (TA1535 or TA1538) and (where appropriate) the rat liver S9 mix were spread over the surface of the bottom agar and the cofactors were incorporated into the top agar. The plates were exposed to a stream of chloroform vapor in an anaerobic jar at 32 ml per hr in triplicate runs. The positive controls, ethyl methanesulfonate (2.5%) and 2-acetylaminofluorene (50 µg/plate) were applied as discs. Negative results were obtained and toxicity was observed after 6-8 hr of exposure to the chloroform vapor. Because the vapor phase study was carried out using a nonstandard procedure, its validity cannot be evaluated. In addition, the positive controls were not volatile and were therefore not tested in the same way as chloroform. Also, only rat liver S9 mix was used for metabolic activation in the vapor phase study, even though the investigators emphasized the advisability of including mouse and rat liver and kidney S9 mixes for the standard protocol.

Gocke et al. (1981) assessed the mutagenicity of 31 chemicals (including chloroform) used as ingredients in European cosmetics. Chloroform was studied in three test systems: the Salmonella/microsome test, the sex-linked recessive lethal test in Drosophila, and the micronucleus test in mice. The latter two tests will be discussed in the following sections. At least five doses, usually up to 3.6 mg/plate for nontoxic and soluble compounds, were tested in the Salmonella/microsome assays. Salmonella strains TA1535, TA100, TA1538, TA98, and TA1537 were used with and without activation by S9 mix prepared from Aroclor-pretreated rats. Three halogenated aliphatic hydrocarbons were tested (1,1,1-trichloroethane, dichloromethane, and chloroform). Gocke and his coworkers placed the plates containing the bacteria, metabolic activation system, and various amounts of the halogenated aliphatic hydrocarbons in airtight desiccators for 8 hr and stated that the desiccators were used because these three substances are volatile. However, this procedure is not likely to prevent evaporation of the volatile test compounds from the plates. The composition and purity of the chemicals were not specified, and details of the protocols were not given. Dichloromethane and trichloroethane exhibited mutagenic activity with and without metabolic activation, but chloroform was inactive. However, information as to whether the cells were exposed to adequate doses of chloroform was not provided, because it is not known whether doses up to a toxic level were tested.

In a screening study of 42 chemicals (de Serres and Ashby, 1981), chloroform was evaluated in 38 in vivo and in vitro short-term tests for potential genotoxicity. Results from bacterial assays carried out in 18 laboratories using Salmonella (Ames reversion test) or E. coli (forward mutation test) were essentially negative. These results are inconclusive, however, because the studies were carried out blind and, therefore, the investigators could not take into account the physicochemical properties (such as volatility) of the substances they were testing when designing their protocols.

Agustin and Lim-Sylianco (1978) investigated the mutagenicity of chloroform in a host-mediated assay in which the indicator organisms were Salmonella strains TA1535 and TA1537 injected into male and female mice. The authors found that male mice metabolized chloroform into a mutagen that was active in strain TA1537. However, only ratios of mutation frequencies for treated and control animals were given, rather than actual colony counts. The ratio of mutation frequencies (tested/control) for strain TA1537 was 36.75 in male mice and 2.30 in female mice. The mutation frequencies for strain TA1535 were 0.61 and 0.12, respectively. The results with strain TA1535 indicate that the mutation frequency of the unexposed bacteria was greater than that of the exposed bacteria. This strange result suggests that the data may be extremely variable. Details of the procedures, such as doses of chloroform, numbers of bacteria injected and recovered, routes of exposure, and times of exposure before the animals were killed, were not presented. Therefore, despite the possibility of a positive response, firm conclusions cannot be reached because of inadequate data presentation, insufficient details about the protocols, and variability of the data.

Agustin and Lim-Sylianco (1978) also studied the mutagenicity of ether-extracted urine concentrates from 10 male mice in a bacterial spot test in strain TA1537. The mice were exposed to chloroform at 700 mg/kg. Urine concentrates from chloroform-treated mice yielded 302 revertant colonies and a zone of inhibition of 29 mm, whereas urine from control animals yielded 10 revertant colonies with no zone of inhibition. Details of the ether extraction procedure were not provided, but the likelihood of a false positive result due to the presence of histidine in the extracted urine is low, because the urine concentrate from the control animals yielded only 10 colonies and was presumably subjected to the same extraction procedure.

In summary, the results of bacterial tests of chloroform are predominately negative, indicating that chloroform is not a mutagen in bacteria. However, false negative results could have been obtained due to a number of factors, including:

1. The activation systems, although adequate for metabolism of the standard positive controls, may have been inadequate for metabolism of chloroform.
2. Phosgene, the primary reactive metabolite of chloroform, is unstable and highly reactive (Kirk-Othmer, 1971). Because exogenous activation systems (i.e., S9 mixes) were used in these negative studies, any phosgene generated may have been scavenged by microsomal protein or lipid before reaching the bacterial DNA. However, the positive result in the urine spot test (Augustin and Lim-Sylianco, 1978), if confirmed, would argue against this. The positive urine spot test suggests that the mutagenic species is stable.
3. Adequate exposure to chloroform may not have occurred if appropriate precautions were not taken to prevent the evaporation of chloroform. The use of a volatile positive control compound would be helpful in this regard. Many of the above studies did not include volatile mutagenic chemicals, either as controls or as additional test compounds. A toxic response at the higher concentrations of chloroform tested would be an indication that the bacteria were adequately exposed.

The positive result in the host-mediated assay cannot be evaluated independently because the details of the procedures and appropriate data were not reported and the data were variable. The results from the urine spot test are suggestive of a positive response. These results indicate a need for additional testing.

7.4. MUTAGENICITY STUDIES IN EUCARYOTIC TEST SYSTEMS

Callen et al. (1980) studied the genetic activity of chloroform in strain D7 of *Saccharomyces cerevisiae*, which contains an endogenous cytochrome P-450 dependent monooxygenase metabolic activation system. By using yeast, Callen and his coworkers eliminated the need for the exogenous type of metabolic activation system used in the bacterial studies. Three different genetic end points can be examined in strain D7: mitotic gene conversion at the *trp5* locus, mitotic crossing over at the *ade2* locus, and reversion at the *ilv1* locus. The effect of chloroform on these end points was measured in cells exposed in suspension to 2.5, 5.0, and 6.3 g of chloroform per liter of buffer (21 mM, 41 mM, and 54 mM, respectively). The purity of the chloroform sample (from J.T. Baker) was not stated. Escape of volatilized chloroform is not expected to have occurred to any significant extent, because the incubations were carried out in screw-capped glass tubes. Results of the study by Callen et al. are presented in Table 7-1. A 1-hr treatment of cells with 54 mM chloroform resulted in an increased ratio of convertants to survivors and a marginal increase in the observed number of convertant colonies. Similar results were obtained for mitotic crossing over and gene reversion. Toxicity at this concentration was high (6% survival).

At the lower concentrations of chloroform (21 mM and 41 mM), a small dose-related increase (1.2-fold and 2.7-fold, respectively) in convertants was observed. In addition, a 9-fold increase in the frequency of genetically altered colonies, which are due primarily to gene conversion and mitotic crossing over, was observed at 41 mM chloroform. For gene reversion, a 2-fold increase was observed at 41 mM chloroform. Toxicity was low at these levels. These results strongly suggest a positive response, but additional studies are needed before it can be stated conclusively that chloroform causes genetic effects in yeast.

TABLE 7-1. GENETIC EFFECTS OF CHLOROFORM ON STRAIN D7 OF S. CEREVISIAE

	Concentration, mM			
	0	21	41	54
Survival				
Total colonies	1423	1302	982	84
% of control	100	91	69	6
trp5 locus (gene conversion)				
Total convertants	246	274	450	278
Convertants/10^5 survivors	1.7	2.1	4.6	33.1
ade2 locus (mitotic crossing over)				
Total twin spots	1	1	2	4
Mitotic cross-overs/10^4 survivors	1.6	1.7	4.1	44.8
Total genetically altered colonies	6	11	43	47
Total genetically altered colonies/ 10^3 survivors	1.0	1.9	8.9	52.7
ilv1 locus (gene reversion)				
Total revertants	61	46	81	50
Revertants/10^6 survivors	4.3	3.5	8.2	60.0

Sturrock (1977) tested the mutagenicity of chloroform at the hypoxanthine- guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster lung fibroblasts (V-79 cells) in culture. The cells were grown to a monolayer and exposed for 24 hr to an atmosphere containing 1 to 2.5% chloroform. Cells were then plated onto media with or without 8-azaguanine. After incubation, theplates were examined for mutations and survival. No significant increase in the frequency of mutants was observed in treated cultures as compared with untreated controls. However, no provision was made for metabolic activation, so the test must be regarded as incomplete.

Gocke et al. (1981) evaluated the mutagenicity of chloroform in the sex-linked recessive lethal test in Drosophila. The flies were exposed by the adult feeding method to 25 mM chloroform. Three successive broods (3-3-4 days) of flies were examined for sex-linked recessive lethal mutations. Over 4000 chromosomes per brood were tested. In two of the broods, small increases in mutations were observed. Frequencies of sex-linked recessive lethals were as follows: Brood 1, 20/4616 chromosomes (0.43%); Brood 2, 13/4349 chromosomes (0.29%); Brood 3, 15/4249 chromosomes (0.35%). Controls were 0.27%, 0.14%, and 0.39%, respectively. Although there may be an increase in the frequency of lethals in Brood 1, this increase was not significant, as determined by the Kastenbaum-Bowman test (Kastenbaum and Bowman, 1970).

Because sperm head abnormalities may be caused by mutations in genes that control spermatogenesis (Wyrobek and Bruce, 1978), assays for morphological sperm abnormalities have been used as indicators of the mutagenic potential of chemicals. In a study of 54 chemicals, Topham (1980) reported that chloroform did not induce sperm head abnormalities in (CBA X BALB/c)F_1 mice. Groups of five male mice received five daily intraperitoneal injections of corn oil alone (5 ml/kg/day) or chloroform in corn oil at 0.025, 0.05, 0.075, 0.1, and 0.25 mg/kg/day. Topham (1980) reported that the highest of these doses (0.25 mg/kg/day) was lethal. Five weeks after the last treatment, caudal sperm smears were examined for morphological abnormalities. Topham (1980) reported that chloroform induced sporadic small increases in abnormal sperm heads at a high dose and that this result could not be repeated. Raw data for the experiments with chloroform were not presented.

A sperm head abnormality test was conducted by Land et al. (1981) in order to determine whether certain anesthetics affect mouse sperm morphology. Groups of five male mice (11 weeks old) were exposed by inhalation to chloroform at 0.04 and 0.08% (vol/vol) for 4 hr/day for 5 days in glass exposure chambers. Control mice (N = 15) were exposed to compressed air under similar conditions. Twenty-eight days after the first exposure, the nine survivors from each exposure level were killed and the caudal sperm were examined for abnormalities. The results were reported as % abnormal sperm (± SEM) and were as follows: control, 1.42 ± 0.08; 0.04% chloroform, 2.76 ± 0.31; 0.08% chloroform, 3.48 ± 0.66. The authors concluded that exposure of mice to chloroform resulted in a significant increase in sperm head abnormalities compared to the control ($\underline{P} < 0.01$). Significance was calculated by the t-test and the F test, but these tests may not be appropriate because of the nonhomogeneity of the variance in chloroform-treated and control groups (Dr. Chao Chen, Carcinogen Assessment Group, U.S. EPA, personal communication). Although this study suggests a positive response, a more appropriate statistical analysis of the data is needed. Unfortunately, the data necessary to carry out the analysis were not provided.

Testing of the mutagenic potential of chloroform in eucaryotic systems was carried out in the same screening study edited by de Serres and Ashby (1981) that was discussed in the previous section for bacterial assays. Seven yeast assays, two in vitro mammalian DNA damage assays (unscheduled DNA synthesis and sister chromatid exchange), and three whole-animal tests (Drosophila sex-linked recessive lethal, mouse bone marrow micronucleus, and mouse sperm abnormality) were reported for chloroform. The DNA damage studies will be discussed in the next section of this chapter.

The seven yeast assays measured forward and reverse mutations, mitotic crossing over, mitotic gene conversion, and aneuploidy in mitotic cells. A positive result was obtained only in a forward mutation assay in Schizosaccharomyces pombe. In the reverse mutation assay, chloroform was tested only in stationary phase cells of S. cerevisiae in the presence of rat S9 mix. Exposure was for 24 hr. Growing cells are more sensitive to the mutagenic effects of several chemicals than are stationary cells, possibly because log-phase yeast cells contain an endogenous cytochrome P-450 metabolizing system (Callen et al., 1980). The negative results in the mitotic gene conversion assay, which was carried out in S. cerevisiae strain D7, are in conflict with the positive results reported for chloroform in strain D7 by Callen et al. (1980). The three whole-animal tests on chloroform yielded negative results (de Serres and Ashby, 1981). Chemical Work Group C in this volume edited by de Serres and Ashby recommended that chloroform be tested further in in vivo short-term tests.

In summary, the results in eucaryotic test systems are primarily negative but inconclusive. More studies, particularly with organisms possessing endogenous activation, are needed before a definite conclusion on the mutagenicity of chloroform can be reached. Suggested studies are described later in this chapter.

7.5. OTHER STUDIES INDICATIVE OF DNA DAMAGE

Two types of DNA damage studies, sister chromatid exchange (SCE) and unscheduled DNA synthesis (UDS), are described in this section.

Sister chromatid exchange (SCE) is widely used as an indicator of induced DNA damage. White et al. (1979) studied the induction of SCEs by chloroform and other anesthetics. Information about the purity of the compounds was not given. Exponentially growing Chinese hamster ovary cells were exposed to the gases in the presence and absence of S9 mix (10% by volume) prepared from Aroclor-induced rat livers. Exposure was at 0.71% (vol/vol) chloroform (88 mM) for 1 hr in closed screw-capped culture flasks. This exposure was 1 human MAC (maximum allowable concentration). After the chloroform was removed from the culture flasks, the cells were incubated for 24 hr in medium containing 10 μM 5-bromo-2'-deoxyuridine (BrdUrd). Numbers of SCEs per chromosome were 0.544 ± 0.018 for chloroform-exposed cells and 0.536 ± 0.018 for the controls. Positive results were observed for anesthetics containing vinyl groups. Although the rat liver S9 was sufficient for activation of vinyl compounds to derivatives (presumably epoxides) that induce SCE, it may not be adequate for activation of chloroform and other haloalkanes. Also, the exposure (1 hr) was short, only one dose was tested, and information on toxicity was not provided.

Another SCE study was carried out by Kirkland et al. (1981) in human lymphocytes. The cells were treated with chloroform at 25, 50, 75, 100, 200, and 400 μg/ml (0.2, 0.4, 0.6, 0.8, 1.6, and 3.3 mM, respectively) for 2 hr in the presence of S9 mix from Aroclor-induced rats. Metaphase spreads from approximately 100 cells per treatment were examined. Acetone was the negative control, and a positive control was not included in the assay because the same donor's lymphocytes had previously shown a dose-related increase in SCE after treatment with benzo(a)pyrene in the presence or absence of S9 mix. A small increase in SCE occurred at 50 µg of chloroform per ml, but no dose-dependent relationship was observed. The study has several shortcomings. First, the positive control was not concurrent. Second, there is no indication that evaporation and escape of chloroform was prevented. Third, the maximal dose was only 3.3 mM. Fourth, information about the toxicity of chloroform for the lymphocytes was not provided.

In contrast to the negative results for the above two SCE tests, Morimoto and Koizumi (1983) found that chloroform and other trihalomethanes induced SCEs in human lymphocytes *in vitro* and mouse bone marrow cells *in vivo*. For the *in vitro* experiment, the lymphocytes were exposed for 72 hr to 0-50 mM chloroform dissolved in dimethyl sulfoxide in the presence of 20 µM BrdUrd. Colcemid (0.2 µM final concentration) was added 3 hr before fixation. Thirty-five second-division cells were scored for each point. A dose-related increase above the control value of 8 SCEs per cell was observed. At 2 mM, 10 mM, and 50 mM chloroform, the numbers of SCEs per cell were about 8.5, 10.5, and 14.7, respectively. The lowest chloroform concentration that caused a significant increase in SCE (Student's *t* test, $P < 0.05$) was 10 mM.

In the *in vivo* experiment, male mice (45-50 g) ingested 0-200 mg of chloroform per kg for 4 days. The chloroform was dissolved in olive oil. Immediately after the last administration, infusion with BrdUrd for 24 hr was initiated. Colcemid (1 mg/kg) was injected intraperitoneally 2 hr before termination of the experiment. The mice were killed and bone marrow cells were removed, fixed in methanol:glacial acetic acid (3:1), and air dried on microslides. The chromosomes were stained by a modification of the fluorescence-plus-Giemsa method. SCEs were analyzed in 25 second-division cells for each animal. A dose-related increase in SCEs per cell was observed. At 0, 25, 50, 100, and 200 mg/kg/day, the mean SCEs per cell were about 5.8, 5.5, 6.8, 7.2, and 9.2, respectively. The lowest chloroform concentration that resulted in a significant increase in SCE (Student's t test, $P < 0.05$) was 50 mg/kg/day.

Two *in vitro* mammalian DNA damage studies on chloroform (UDS and SCE) were described in the volume edited by de Serres and Ashby (1981). The SCE assay (Chapter 51) utilized an exogenous activation system and yielded negative results. Chinese hamster ovary cells were exposed to chloroform (0.084 to 84 μm) in the presence of rat liver S9 mix for 1 hr. This treatment time may be insufficient, particularly since a positive response for 2-acetylaminofluorene in the presence of S9 was obtained after a 2-hr exposure but not after a 1-hr exposure. In addition, the cells were exposed to very low levels of chloroform and there was no indication that precautions were taken to prevent evaporation and loss of chloroform from the culture flasks. Thus, the negative results obtained in this study are inconclusive.

Unscheduled DNA synthesis (UDS), which is a measure of DNA repair, can be used as an indicator of chemically induced DNA damage. Mirsalis et al. (1982) measured UDS in primary rat hepatocyte cultures following *in vivo* treatment of adult male Fischer-344 rats (175-275 g) with chloroform at 40 and 400 mg/kg by gavage. Control rats received corn oil (the vehicle for chloroform) by gavage. At 2 or 12 hr after treatment, the livers were perfused *in situ* and hepatocytes were isolated. Approximately 6×10^5 viable cells were seeded in 35-mm culture dishes containing coverslips and allowed to attach to the coverslips for about 90 min. After the coverslip cultures were washed, they were incubated in a medium containing 10 μCi [^{3}H]thymidine (40-50 Ci/mmol) per ml for 4 hr. The cultures were washed again and incubated in medium containing 0.25 mM cold thymidine for 14-16 hr. The extent of UDS was assessed by autoradiography. Net grains/nucleus were calculated as the silver grains over the nucleus minus the highest grain count of three adjacent nuclear-sized areas over the cytoplasm.

Cells from negative control animals (given vehicle only) ranged from -3.0 to -5.1 net grains/nucleus. Several chemicals were positive in this assay (≥5 net grains/nucleus was considered positive), including methyl methanesulfonate, dimethylnitrosamine, 2-acetylaminofluorene, and benzidine. Chloroform at 40 and 400 mg/kg yielded a negative response (-2.7 to -4.4 net grains/nucleus). However, rats are not susceptible to chloroform-induced hepatocarcinogenesis (NCI bioassay, 1976). Benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene, carcinogens that, like chloroform, are not rat liver carcinogens, were also negative in this assay. These chemicals were positive, however, in the *in vitro* rat hepatocyte UDS assay (Williams, 1981). This discrepancy suggests that the *in vitro* test may be more sensitive than the *in vivo* assay. Chloroform has not been tested in the *in vitro* rat hepatocyte UDS assay. Since the mouse is susceptible to chloroform-induced liver tumors (NCI bioassay, 1976), it may be a more appropriate test animal than the rat for the *in vivo* UDS assay.

It is uncertain whether measuring UDS by subtraction of cytoplasmic grain counts from nuclear grain counts would allow for detection of a weak response. In a recent article discussing the validity of the autoradiographic procedure for detecting UDS in rat hepatocytes, Lonati-Galligani et al. (1983) describe some potential problems with this method. First, they found that it is difficult to obtain hepatocyte preparations of reproducible quality. Since preparations can differ in their metabolic capabilities, they suggest that test chemicals should be studied in conjunction with a potent UDS-inducing analog and that negative results be accepted only in tests in which the analog is strongly positive. No known positive analog of chloroform was tested in the study of Mirsalis et al. (1982). Second, the cytoplasmic layer covering the nucleus is thinner than the cytoplasmic area next to the nucleus. Therefore, a variable overcorrection is probably applied, as witnessed by the usually higher cytoplasmic than nuclear counts observed in control cells. This effect would tend to obscure a weakly positive UDS response. Lonati-Galligani et al. (1983) suggest that the grains over the nucleus and over a cytoplasmic area should be scored and dose-response curves plotted separately instead of subtracting cytoplasmic grain counts from nuclear grain counts. Both dose-response curves should be considered before a decision is reached on whether exposure to a certain chemical results in UDS.

Results of an additional DNA repair study were published by Reitz et al. (1980a). Mice were exposed orally to chloroform at 240 mg/kg, and DNA repair in the livers was assayed. Negative results were obtained. The methodology used to assay for DNA repair was provided in a paper on vinylidine chloride from the same laboratory (Reitz et al., 1980b). The difference in the control values in the vinylidine chloride paper was an order of magnitude in some cases, indicating that the assay methodology was not sensitive enough to pick up repair DNA synthesis. Neither paper included information on the length of time the mice were exposed to chloroform. Some compounds require a longer exposure than others, as evidenced by the results with 2-acetylaminofluorine in Mirsalis et al. (1982). The possibility of false negative results, as discussed above, exists in this study as well.

The UDS assay discussed in Chapter 48 of the de Serres and Ashby (1981) volume was carried out with HeLa cells, which do not contain a cytochrome P-450 activation system. An exogenous rat liver S9 activation system was employed. Although chloroform was positive in this assay in the absence of the activation system, the discussion of this assay in the de Serres and Ashby book suggests that the result may be misleading because of inadequate statistical evaluation. In the presence of rat liver S9, chloroform was negative.

In summary, many of the above studies on the DNA-damaging potential of chloroform suffer from various deficiencies. Three negative SCE studies were inconclusive. Positive results for in vitro and in vivo SCE were reported by Morimoto and Koizumi (1983). Three negative UDS studies were inconclusive. Increases in mitotic gene conversion and mitotic crossing over in yeast suggest that chloroform damages DNA (Callen et al., 1980). The weight of the available evidence suggests that chloroform may damage DNA. Additional studies will be required, however, before firm conclusions can be reached.

7.6. CYTOGENETIC STUDIES

Kirkland et al. (1981) studied the ability of chloroform to induce chromosome breakage in cultured human lymphocytes. The cells from one donor were treated with chloroform at 50, 100, 200, and 400 μg/ml for 2 hr in the presence of an S9 activation system derived from Aroclor 1254-induced rats. The positive control compound, benzo(a)pyrene, in a separate experiment with the same donor's lymphocytes induced chromosome breakage with or without S9 treatment. The response of this donor's lymphocyte chromosomes to chloroform was a random variation around the control value. The highest breakage level was at 200 μg/ml with 8 breaks/100 cells compared with 5.5 breaks/100 cells in the control. However, this difference was not significant in a chi-square test. The same problems discussed in the previous section for the SCE study carried out by Kirkland et al. (1981) apply to their study on chromosome breakage.

According to Schmid (1976), the bone marrow micronucleus test can be used to detect clastogens and spindle poisons. Micronuclei are small nucleus-like elements that contain either chromosomal fragments that originated from clastogenic events or whole chromosomes that were segregated by malfunction of the spindle apparatus. Gocke et al. (1981) tested chloroform in a micronucleus test in mice. The animals were treated with chloroform at 0 and 24 hr, and bone-marrow smears were prepared at 30 hr. The purity of the chloroform (purchased from Merck) was not stated. Four mice (two males and two females) were used for each of three doses of chloroform and for an untreated control group. The animals were each given two intraperitoneal injections of chloroform for individual treatment dosages of 238, 476, and 952 mg/kg (2, 4, and 8 mmol/kg). Since the assay was performed by the method of Schmid (1976), one would assume that the treatments included the maximum tolerated dose. However, details of dosage selection were not discussed. Slides were coded, and 1000 polychromatic erythrocytes were scored per mouse.

The results were as follows (dose, number of micronucleated polychromatic erythrocytes per 1000 polychromatic erythrocytes): 0 mg/kg, 1.2; 2 x 238 mg/kg, 2.2; 2 x 476 mg/kg, 2.6; 2 x 952 mg/kg, 2.2. Although the values for the treated groups were higher than the control, the results were not significant at $\underline{P} < 0.01$ according to the Kastenbaum-Bowman test (Kastenbaum and Bowman, 1970). Three halogenated alkanes were tested (dichloromethane, 1,1,1-trichloroethane, and chloroform), and all yielded negative results. Of 30 chemicals tested, only two (pyrogallol and hydroquinone) yielded positive results in the micronucleus test. Positive controls were not included in the assay, but the positive results for pyrogallol and hydroquinone indicate that the assay system was working.

The micronucleus test was also used by Agustin and Lim-Sylianco (1978) to study the clastogenic potential of chloroform. The authors tested seven concentrations of chloroform up to 900 mg/kg in the mouse. The number of mice used and their sex were not specified. The chloroform was purchased from Mallinckrodt and was redistilled before use. For each slide, 1000 polychromatic erythrocytes were scored. The authors reported that chloroform was clastogenic. Results were as follows (dose in mg/kg, number of micronucleated polychromatic erythrocytes per 1000 polychromatic erythrocytes ± SE): 0, 4 ± 1; 100, 3 ± 1; 200, 5 ± 1; 400, 5 ± 1; 600, 9 ± 2; 700, 17 ± 4; 800, 9 ± 2; 900, 10 ± 2. The authors interpreted the nonlinearity of the dose-response relationship as evidence that chloroform must be metabolized to a clastogenic substance. The data, however, are not sufficient to reach any conclusions regarding the metabolism of chloroform. In the same paper, Agustin and Lim-Sylianco demonstrated that vitamin E administered 1 hr after chloroform reduced the number of micronucleated cells observed at 700 mg of chloroform/kg (17 ± 4) to the control level (4 ± 1). The significance of this result is not clear.

The paper by Agustin and Lim-Sylianco gives insufficient details of the experimental procedures for an independent evaluation of the results. For example, the number and sex of the animals and positive control data are not given. This study suggests that chloroform may cause chromosome damage, but corroborative studies are needed to confirm or refute this interpretation.

A cytogenetic assay for mitotic poisons in grasshopper embryos indicates that chloroform vapors induce a complete arrest of cells at metaphase (Liang et al., 1983). Grasshopper embryos (eggs) at 7-9 days of development were suspended in 95-ml jars and chloroform (MCB Reagents) at 0.01 (30,000 ppm), 0.05, 0.1, and 0.2 ml per jar was placed on the bottom of the jars. The jars were closed with screw caps and tightly sealed with petroleum jelly. Embryos (12 total) exposed to air alone in jars served as untreated controls. Three randomly selected embryos were exposed in each jar. The jars were incubated at 24°C for 16 hr. During this time, the chloroform completely vaporized and the embryos were exposed to the chloroform vapor through respiration. After treatment, the embryos were made into squash preparations. In each squash preparation, the mitotic index (MI) and the anaphase to metaphase ratio (A/M) were determined. MI in each embryo is the percentage of mitoses (metaphases and anaphases) estimated by scanning 3000 cells, and A/M is the number of anaphases present per 100 metaphases. Statistical significance was determined by chi square analysis. If chloroform completely arrested mitosis at metaphase, the A/M would be zero; if chloroform does not prevent interphase cells from entering mitosis, the MI would be elevated compared to the control.

The results shown in Table 7-2 indicate that chloroform arrested mitosis at metaphase. In control embryos, all stages of mitosis were observed; the average MI was 1.0 and the average A/M was 0.65. At a chloroform dose of 0.05 ml per jar, the average A/M was zero and the average MI was 11 times that of the control. The arrested metaphases showed colchicine-like mitotic effects (c-mitosis), such as lagging chromosomes and multipolar spindles. Liang et al. (1983) concluded that chloroform interferes with spindle microtubules, thereby causing mitotic arrest at metaphase.

TABLE 7-2. MITOTIC INDEX (MI), ANAPHASE/METAPHASE (A/M), AND PRESENCE (+) OF COMPLETE C-MITOSIS IN GRASSHOPPER EMBRYOS AFTER EXPOSURE TO $CHCl_3$ VAPOR

Amount of $CHCl_3$ per jar (ml)	MI	A/M	Complete c-mitosis
Untreated control	1.0 (0.7 - 1.6)[a]	0.65 (0.48 - 1.33)	
0.01	1.2[b]	0.49	
0.05	11.1*	0.*	+
0.1	6.4*	0.*	+
0.2	-[c]	-	

[a] Average of 12 embryos (range).
[b] Average of 3 embryos at each concentration.
[c] Embryo death induced.
*Significantly different from control, $p < 0.005$.

SOURCE: Liang et al. (1983)

In summary, of the three available studies on the clastogenic effects of chloroform, one was negative, one was negative but inconclusive, and one was positive but inconclusive. More studies are needed before a conclusion is reached as to whether chloroform is clastogenic. The data reported by Liang et al. (1983) suggest that chloroform affects spindle microtubules.

7.7. SUGGESTED ADDITIONAL TESTING

Additional studies are needed to measure covalent binding of $^{14}CHCl_3$ to DNA. The ability of chloroform to cause DNA damage should be studied further in UDS and SCE tests. Suggested testing includes measurement of UDS *in vivo* in mice and *in vitro* in both rat and mouse hepatocytes. The issues raised by Lonati-Galligani et al. (1983) should be taken into consideration when choosing procedures for the UDS assays. Additional studies are needed to corroborate or refute the study of Callen et al. (1980) in yeast. Further testing for the ability of chloroform to cause changes in chromosome structure and number is needed, particularly in *in vivo* systems.

7.8. SUMMARY AND CONCLUSIONS

It has been demonstrated that chloroform can be metabolized in vivo and in vitro to a substance (presumably phosgene) that interacts with protein and lipid. The potential for metabolically activated chloroform to bind to DNA cannot be determined from the available studies, but, if binding to DNA does occur, it would be at a very low level.

The majority of the assays for genotoxicity have yielded negative results. However, many of these results are inconclusive because of inadequacies in the experimental protocols. The major problem has been the use of exogenous activation systems (i.e., S9 mix). In none of the studies was it shown that chloroform was activated or metabolized by the activation system used. Metabolism of 2-aminoanthracene or vinyl compounds (used as positive controls) is an inadequate indication that the activation system can metabolize chloroform, because these substances are not halogenated alkanes. A better indication that the activation system is sufficient for metabolism of chloroform may be to show that it metabolizes $^{14}CHCl_3$ to intermediates that bind to macromolecules. A second problem in the use of exogenous activation systems is the possibility that highly reactive metabolites may react with microsomal or membrane lipid or protein before reaching the DNA of the test organism. Another problem in in vitro tests is that adequate precautions are sometimes not taken to prevent the escape of volatilized chloroform.

On the basis of presently available data, no definitive conclusion can be reached concerning the mutagenicity of chloroform. However, evidence from studies measuring binding to macromolecules, DNA damage, and mitotic arrest suggest that chloroform may be mutagenic. Alternatively, because recent studies on the mechanism of action of tumor promoters suggest that promoters can damage DNA [see Marx (1983) for review], chloroform may promote carcinogenesis rather than initiate it.

7.9 REFERENCES FOR CHAPTER 7

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8. CARCINOGENICITY

8.1. ANIMAL STUDIES

The carcinogenicity of chloroform has been evaluated in mice, rats, and dogs. Evidence for carcinogenic activity by chloroform includes induction of renal epithelial tumors, mostly malignant, in male Osborne-Mendel rats (National Cancer Institute [NCI], 1976 and Jorgenson et al.,1985), hepatocellular carcinomas in male and female B6C3F1 mice (NCI, 1976), kidney tumors in male ICI mice (Roe et al., 1979), and hepatomas in female strain A mice (Eschenbrenner and Miller, 1945) and NLC mice (Rudali, 1967). Capel et al. (1979) demonstrated an ability of chloroform to promote growth and metastasis of murine tumors. Chloroform was not shown to be carcinogenic in (C57 x DBA2 F1) mice (Roe et al., 1968), female Osborne-Mendel rats (NCI, 1976), female ICI mice and male mice of the CBA, C57BL, and CF/1 strains (Roe et al., 1979), female B6C3F1 mice when administered in drinking water (Jorgenson et al., 1985), male and female Sprague-Dawley rats (Palmer et al., 1979), and male and female beagle dogs (Heywood et al., 1979). Chloroform was negative in a pulmonary tumor induction bioassay in male strain A/St mice (Theiss et al., 1977). Chloroform in liquid solution did not induce transformation of baby Syrian hamster kidney cells (BHK-21/C1 13) *in vitro*. Under the conditions of the carcinogenicity bioassays showing carcinogenic activity for chloroform specifically in kidney and liver of mice and rats, the conclusion can be made, by applying either the EPA proposed Carcinogen Risk Assessment Guidelines or the IARC classification approach for carcinogens, that there is sufficient evidence for the carcinogenicity of chloroform in experimental animals.

8.1.1. Oral Administration (Gavage): Rat

8.1.1.1. National Cancer Institute (1976)--A carcinogenesis bioassay on chloroform in Osborne-Mendel rats was reported by the NCI (1976). The chloroform product (Aldrich Chemical Company, Milwaukee, Wisconsin) was analyzed at the carcinogenesis bioassay laboratory and was shown by gas-liquid chromatography, flame ionization detection, and infrared spectrometry to be 98 percent pure chloroform and 2 percent ethyl alcohol (stabilizer). Fresh chloroform solutions in corn oil were prepared each week and stored under refrigeration.

Fifty animals of each sex were randomly assigned to each of two chloroform dose test groups. Treated animals were compared with matched vehicle-control groups (20 males and 20 females) and with a vehicle colony control group (99 males and 98 females). This latter group included the matched control group and three other control groups put on study within 3 months of the matched control group. Matched control and treated animals were housed in the same room, and colony controls were housed in two different rooms.

Doses selected for the long-term carcinogenicity study in rats were estimated as the doses maximally tolerated by the animals and one-half the maximally tolerated doses based on survival, body weights, clinical signs, and necropsy examinations in a preliminary toxicity test in each sex. The chloroform was given by gavage for 6 weeks followed by an observation period of 2 weeks without treatment. The chronic study began with 52-day-old rats and ended with sacrifice of the survivors at 111 weeks. Chloroform was administered to the rats in corn oil by gavage 5 days each week during the initial 78 weeks. Doses of 90 and 180 mg/kg/day were administered to male rats throughout the chronic study; however, since initial doses of 125 and

250 mg/kg/day were reduced to 90 and 180 mg/kg/day at 22 weeks, doses given to female rats were expressed as time-weighted averages of 100 and 200 mg/kg/day.

Body weights and food consumption were monitored weekly for the first 10 weeks and monthly, thereafter. Animals were observed twice daily. Decedents and survivors were necropsied, and tissues and organs were examined microscopically.

In matched control and both dose groups, at least 50 percent of the male and female rats survived as long as 85 and 75 weeks, respectively. Seven matched control, 24 low-dose, and 14 high-dose males, and 15 matched control, 22 low-dose, and 14 high-dose females survived until the end of the study. Only one control male rat died before 90 weeks; the increase in death rate of control males after 90 weeks was, according to the NCI (1976) report, "probably due to respiratory and renal conditions." Overall survival was reduced in treated females and high dose males relative to controls (Figure 8-1).

Appearance and behavior among groups were generally similar, but hunching, urine stains on the lower abdomen, redness of eyelids, and wheezing were noted in treated animals early in the study. Food consumption was reported as slightly less in treated animals, but data were not provided. Decreased body weight gain was evident in both treatment groups in both sexes of rats. Initial mean body weights for all groups were about 175 g for females and 250 g for males. By 50 weeks, mean body weights were approximately 400 g in control, 350 g in low-dose, and 330 g in high-dose females; by 100 weeks, mean body weights were about 375 g in all groups of females. In males, mean body weights were about 640 g in the control group,

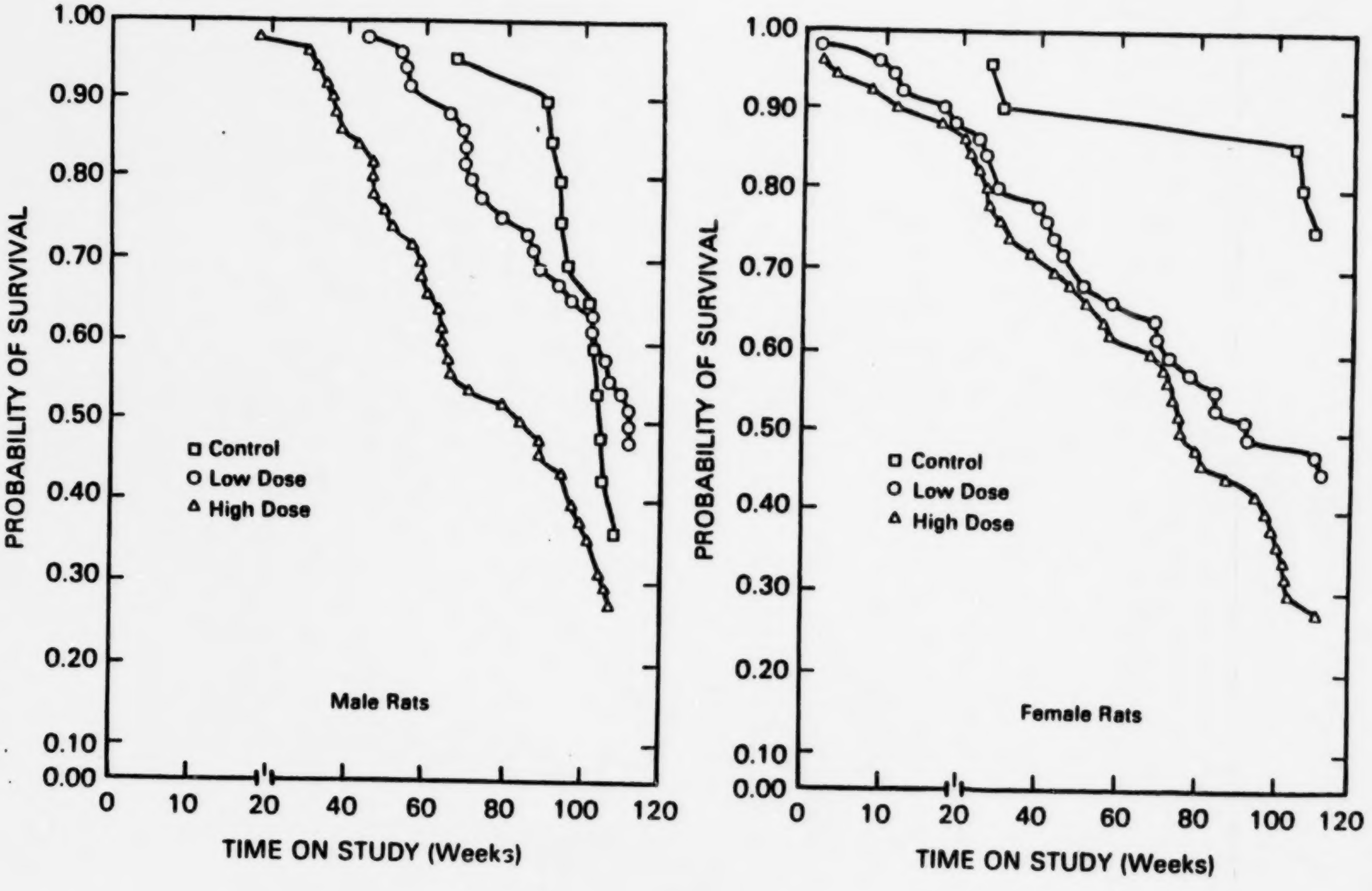

Figure 8-1. Survival curves for Fischer 344 rats in a carcinogenicity bioassay on chloroform.

Source: NCI (1976).

550 g in the low-dose group, and 500 g in the high-dose group by 50 weeks; by 100 weeks, mean body weights were approximately 500 g in all groups.

A statistically significant ($P < 0.05$) dose-related increase in renal epithelial tumors of tubular cell origin was found in treated male rats (Table 8-1). The epithelial tumors were described as follows: Of 13 tumors in high-dose males, 10 were carcinomas and three were adenomas; two carcinomas and two adenomas comprised the tumors found in four low-dose males; one renal epithelial carcinoma and one squamous cell carcinoma from renal pelvic transitional epithelium were noted in two high-dose females. One low-dose male had both a malignant mixed tumor and a tubular cell adenoma in the left kidney, and a high-dose male had a tubular cell carcinoma and a tubular cell adenoma in the right kidney. Renal epithelial carcinomas were large and poorly circumscribed, and they infiltrated surrounding normal tissue. Renal epithelial adenomas were circumscribed and well-differentiated. Additional kidney tumors included malignant mixed tumors in two low-dose and two colony control males and hamartomas in one low-dose male, one high-dose male, and one colony control male.

Although a statistically significant ($P < 0.05$) increase in thyroid tumors was reported in both treatment groups of female rats as compared with colony controls, the toxicologic significance of this finding is controversial because C-cell tumors and follicular cell tumors, which have different embryonic origins and different physiologic functions, are combined in the incidences described in Table 8-2; the majority of tumors were adenomas; the spontaneous incidence of thyroid tumors in Osborne-Mendel females is variable as stated, in the NCI (1976) bioassay report although without presentation of historical data; and the increased incidence of thyroid tumors in treated females is not statistically significant ($P > 0.05$)

TABLE 8-1. EFFECT OF CHLOROFORM[a] ON KIDNEY EPITHELIAL TUMOR INCIDENCE IN OSBORNE-MENDEL RATS (NCI, 1976)

	Male				Female			
	Controls[b]		Dose (mg/kg/day)[c]		Controls[b]		Dose (mg/kg/day)[c]	
Treatment	Colony	Matched	90	180	Colony	Matched	100	200
Kidney tumor incidence[d]	0/99(0%)	0/19(0%)	4/50(8%)[e]	12/50(24%)[f]	0/98(0%)	0/20(0%)	0/49(0%)	2/48(4%)[g]
P value[h]			0.266	0.014[i]			--	0.495
Time to first tumor (weeks)	---	---	102	80	---	---	---	102
Survival at terminal sacrifice (111 weeks)	26%	37%	48%	28%	51%	75%	45%	29%

[a] Chloroform in corn oil administered by gavage 5 times per week for 78 weeks.
[b] Colony controls consist of four vehicle-control groups, including matched controls, given corn oil.
[c] Doses are time-weighted averages.
[d] Animals with tumor/animals examined.
[e] Two with tubular cell adenocarcinoma and two with tubular cell adenoma.
[f] Ten with tubular cell adenocarcinoma and two with tubular cell adenoma.
[g] One with tubular cell adenocarcinoma and one with squamous cell carcinoma in the renal pelvis.
[h] Fisher's Exact Test, compared with matched controls.
[i] For adenocarcinomas alone, P value is 0.03.

TABLE 8-2. EFFECT OF CHLOROFORM ON THYROID TUMOR INCIDENCE IN FEMALE OSBORNE-MENDEL RATS (NCI, 1976)

Dose[a,b] (mg/kg/day)	Follicular cell tumors[c]	C-cell tumors[d]	Total tumors[e]		
	Incidence[f]	Incidence	Incidence	P value[g]	Time to first tumors (weeks)
0 (matched)[h]	1/19(5%)	0/19(0%)	1/19(5%)		110
0 (colony)[h]	1/98(1%)	0/98(0%)			110
100	2/49(4%)	6/49(12%)	8/49(16%)	0.216	73
200	6/49(12%)	4/49(8%)	10/49(20%)	0.121	49

[a] Chloroform in corn oil administered by gavage 5 times per week.
[b] Time-weighted average doses.
[c] Adenomas except for carcinoma in one low-dose and two high-dose animals.
[d] Adenomas except for carcinoma in one high-dose animal.
[e] See text.
[f] Animals with tumors/animals examined.
[g] Fisher's Exact Test, compared with matched controls.
[h] Colony controls consist of four vehicle-control groups, including matched controls, given corn oil.

when compared with data for the matched controls only. No significant ($P < 0.05$) differences for other tumor types were apparent among the groups of rats. Four rats were lost (missing or autolyzed) for pathology.

Non-neoplastic lesions described as treatment-related include necrosis of liver parenchyma, epithelial hyperplasia in the urinary bladder, and hematopoiesis in spleen. Inflammatory pulmonary lesions characteristic of pneumonia were found in all groups, but the severity and incidence of these lesions were stated (data not reported) to have been greater in treatment groups.

Under the conditions of this bioassay, chloroform treatment significantly ($P < 0.05$) increased the incidence of renal epithelial tumors in male Osborne-Mendel rats. Although the number of matched vehicle controls was low, the use of pooled colony controls gives additional support for treatment-related effects. Moreover, historical control incidence of renal epithelial tumors in Osborne-Mendel rats was reported as rare.

Lower survival rates and body weights in treated rats than in matched controls provide evidence that the chloroform doses used may have been toxic to the rat strain used in this study. A more precise estimate of dose-response perhaps could have been obtained if additional lower doses had been given, and if constant doses rather than time-weighted averages had been used for the females as was done for the males. Treated animals were housed in the same room as rats treated with other volatile compounds (1,1,2,2-tetrachloroethane, 3-chloropropene, ethylene dibromide, carbon tetrachloride); however, since controls were in the same room as treated animals, and oral chloroform doses probably were much higher than ambient levels of other volatiles, the likelihood that the other volatile compounds were responsible for the observed results is considered to be low.

Additionally, these other volatile compounds did not induce kidney tumors in Osborne-Mendel rats (NCI, 1976; Weisburger, 1977). It should be noted that ambient levels of volatiles in the animal quarters were not measured.

8.1.1.2. Palmer et al. (1979)--Palmer et al. (1979) reported carcinogenicity studies on chloroform in Sprague-Dawley rats. The chloroform dose preparations were concocted in toothpaste, as described in Table 8-3 herein for the Roe et al. (1979) study, and administered by gavage. Dose levels of 15, 75, and 165 mg $CHCl_3$/kg/day were selected for the carcinogenicity study based on results of a preliminary range-finding study suggesting the lowest toxic dose, indicated by liver and kidney changes, as 150 mg/kg/day.

TABLE 8-3. TOOTHPASTE FORMULATION FOR CHLOROFORM ADMINISTRATION
(Roe et al., 1979)

Ingredient	Percentage w/w
Chloroform	3.51
Peppermint oil[a]	0.25
Eucalyptol[a]	0.50
Glycerol	39.35
Carragheen gum	0.45
Precipitated calcium carbonate	48.53
Sodium lauryl sulphate	1.16
Sodium saccharin	0.03
White mineral oil	1.10
Water	5.12
Total	100.00

[a] Essential oil flavor components.

An initial carcinogenicity study was conducted in which 25 rats of each sex per group received one of the selected doses in toothpaste containing essential oils (flavor components), indicated in Table 8-3, 6 days per week. A concurrent control group of 75 males and 75 females was administered toothpaste without chloroform or essential oils. A second carcinogenicity study was conducted in which 50 male and 50 female specific pathogen-free (SPF) Sprague-Dawley rats were dosed with 60 mg chloroform/kg/day in toothpaste with essential oils 6 days per week, and 50 control rats of each sex were given toothpaste without chloroform but with essential oils.

Body weights were measured weekly, and food consumption was recorded. Body weights were initially 180 to 240 g for males and 130 to 175 g for females. Blood and urine analyses were performed in the first study, and serum and erythrocyte cholinesterase activities and other serum enzyme activities were monitored in the second study. All animals were necropsied, and tissues and organs were examined histopathologically. Adrenal glands, kidneys, livers, lungs, and spleens were weighed.

Chloroform was not carcinogenic in these studies. Significant ($P < 0.05$) body weight loss in high-dose males in the first study (data not reported) and maximal body weight gain of approximately 370 g in control males, 330 g in treated males, 220 g in control females, and 180 g in treated females in the second study suggest an effect from chloroform treatment. Other than a 40 percent reduction of plasma cholinesterase levels and slight decreases in serum glutamic-pyruvic transaminase and serum alkaline phosphatase in treated females, additional toxic effects from chloroform treatment were not evident.

Low survival, attributed to respiratory disease, was apparent in both studies. The initial study was terminated at 52 weeks; 50 percent of the animals in all groups had died by 52 weeks in the second study, which was ended at 95 weeks. Except for 48 control females in the initial study, no more than 18 animals were alive in each group at the conclusion of either study. Although carcinogenic activity for chloroform was not observed, these studies on Sprague-Dawley rats are weakened by the high early mortality in both control and treated animals.

8.1.2. Oral Administration (Gavage): Mouse

8.1.2.1. National Cancer Institute (1976)--The carcinogenicity of chloroform in B6C3F1 mice was evaluated by the NCI (1976). The chloroform product and chloroform solutions in corn oil were those used in the NCI (1976) carcinogenesis bioassay in rats previously described in Section 8.1.1.1.

Each of two dose treatment groups was composed of 50 males and 50 females. Treated mice were compared with matched vehicle control groups (20 males and 20 females) put on study 1 week earlier, and with vehicle colony control groups (77 males and 80 females), which included the matched control group and three other control groups put on study within 3 months of the matched control group. All control mice were housed in the same room with treated mice.

Maximally tolerated doses and one-half the maximally tolerated doses for the long-term carcinogenicity study in mice were estimated from a preliminary toxicity test conducted as described for the NCI (1976) rat study. Mice were 35 days of age at the start of the chronic study, and the study was concluded with the sacrifice of survivors at 92-93 weeks. Chloroform in corn oil was administered by gavage 5 days per week during the first 78 weeks. Initial dose levels of 200 and 100 mg/kg/day for males and 400 and 200 mg/kg/day for females were raised to 300 and 150 mg/kg/day for males and 500 and 250 mg/kg/day for females at 18 weeks. Thus, doses expressed as time-weighted averages for the entire study were 138 and 277 mg/kg/day for males and 238 and 477 mg/kg/day for females.

Survival in mice was similar among groups except for high-dose females. At least 50 percent of the animals in each group survived as long as 85 weeks. Ten matched control, 33 low-dose, and 30 high-dose males, and 15 matched control, 34 low-dose, and 9 high-dose females survived for the duration of the study. All but two deaths in high-dose females occurred after 70 weeks.

Body weight gain among groups was comparable. Male and female mice initially weighed about 18 and 15 g, respectively. Mean body weights at 50 weeks were approximately 35 g in males and 28 g in females, and these levels were generally sustained throughout the remainder of the study. Food consumption was stated to have been equivalent among groups. Appearance and behavior among groups were similar except for bloating and abdominal distension noted in treated animals beginning after 42 weeks of treatment.

Statistically significant ($P < 0.05$) dose-related increases in hepatocellular carcinomas in both treatment groups in both sexes of mice were observed (Table 8-4). Various histopathologic types of hepatocellular carcinomas were observed. The hepatocellular carcinoma metastasized to the lung in two low-dose males and two high-dose females, and to the kidney in one high-dose male. Twenty animals were reported as missing or autolyzed, and therefore were not included in the pathology report.

Nonneoplastic lesions in mice attributed to treatment were relatively few. These include nodular hyperplasia of the liver in 10 low-dose males, six low-dose females, and one high-dose female; and liver necrosis in one low-dose male, four low-dose females, and one high-dose female. Nine high-dose females with hepatocellular carcinoma had cardiac atrial thrombosis.

TABLE 8-4. EFFECTS OF CHLOROFORM[a] ON HEPATOCELLULAR CARCINOMA INCIDENCE IN B6C3F1 MICE (NCI, 1976)

	Male				Female			
	Controls[b]		Dose (mg/kg/day)[c]		Controls[b]		Dose (mg/kg/day)[c]	
Treatment	Colony	Matched	138	277	Colony	Matched	238	477
Hepatocellular carcinoma incidence[d]	5/77(8%)	1/18(6%)	18/50(36%)	44/45(98%)	1/80(1%)	0/20(0%)	36/45(80%)	39/41(95%)
P value[e]			0.011	3.13×10^{-13}			4×10^{-10}	3.7×10^{-14}
Time to first tumor (weeks)	72	72	80	54	90	---	66	67
Survival at terminal sacrifice (111 weeks)	48%	50%	65%	65%	81%	75%	75%	20%

[a] Chloroform in corn oil administered by gavage 5 times per week for 78 weeks.
[b] Colony controls consist of four vehicle-control groups, including matched controls, given corn oil.
[c] Doses are time-weighted averages.
[d] Animals with tumor/animals examined.
[e] Fisher's Exact Test, compared with matched controls.

Kidney inflammation was diagnosed in 10 matched control males, two low-dose males, and one high-dose male.

Under the conditions of this bioassay, chloroform treatment significantly ($P < 0.05$) increased the incidence of hepatocellular carcinoma in male and female B6C3F1 mice. Although the number of matched vehicle controls was low, the use of pooled colony controls gives additional support for treatment-related effects. Moreover, historical control incidence of hepatocellular carcinomas in B6C3F1 mice was reported as 5-10 percent in males and 1 percent in females.

A more precise estimate of dose-response perhaps could have been obtained if additional lower doses had been used and if constant doses rather than time-weighted averages had been used. Treated animals were housed in the same room as animals treated with other volatile compounds*; however, since 1) controls were in the same room as treated animals, 2) oral chloroform doses probably would have been much higher than ambient levels of other volatiles, 3) the cages had filters to limit the amount of chemical released into the ambient air, 4) the total room air was exchanged 10 to 15 times per hour, and 5) dosing was done in another room under a large hood, the likelihood that the other volatile compounds were responsible for the observed results is considered to be low. It should be noted that ambient levels of volatiles in the animal quarters were not measured.

8.1.2.2. Roe et al. (1979)--Roe et al. (1979) studied the carcinogenicity of chloroform in toothpaste in four strains of mice (C57BL, CBA, CF/1, and ICI).

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*1,1,2,2-tetrachloroethane, 3-chloropropene, chloropicrin, 1,1-dichloroethane, trichloroethylene, sulfolene, iodoform, ethylene dichloride, methyl chloroform, 1,1,2-trichloroethane, tetrachloroethylene, hexachloroethane, carbon disulfide, trichlorofluoromethane, carbon tetrachloride, ethlene dibromide, dibromochloropropane.

The toothpaste formulation used is presented in Table 8-3. The chloroform product was described as British Pharmacopoeia grade which was not contaminated with other haloalkanes or phosgene. Toothpaste was prepared fresh each month. Chloroform in arachis oil vehicle was also tested in ICI mice.

Dose levels for the carcinogenicity studies were selected based on results of a 6-week preliminary range-finding study in male and female Schofield mice which indicated moderate weight gain reduction at the lowest dose considered toxic, which was 60 mg $CHCl_3$/kg/day. Three different carcinogenicity studies were conducted in which mice, initially no more than 10 weeks old, were given chloroform by gavage 6 days per week for 80 weeks followed by observation for 13 to 24 weeks. In one study, 52 male and 52 female ICI mice per dose group were given 17 or 60 mg/kg/day of chloroform in toothpaste and compared with 100 ICI mice of each sex concurrently given toothpaste without chloroform, peppermint oil, or eucalyptol. A second study, confined to male ICI mice, included 52 untreated mice, 260 mice given toothpaste alone without chloroform, eucalyptol, or peppermint oil, and groups of 52 mice each given, in toothpaste, 60 mg $CHCl_3$/kg/day, eucalyptol up to 32 mg/kg/day, or peppermint oil up to 16 mg/kg/day; treatment with chloroform, eucalyptol, or peppermint oil was performed in the absence of the other two compounds. In the third study, groups of 52 male mice of each of the C57CL, CBA, CF/1, and ICI strains were given 60 mg $CHCl_3$/kg/day in toothpaste and compared with concurrent vehicle-control groups of 52 mice each, and with 100 untreated ICI mice. Fifty-two ICI male mice given 60 mg $CHCl_3$/kg/day in arachis oil, and concurrent control mice given arachis oil alone, were also evaluated in the third study.

Body weights were recorded in each study, and food consumption was estimated in the second and third studies. In each study, the animals were necropsied, and tumors and other lesions as well as routine tissues and organs were examined histopathologically. Adrenal glands, kidneys, livers, lungs, and spleens were weighed.

Although the authors stated (data were not reported) that body weight gain was poorer in each treatment group than in controls in the third study on the four mouse strains, differences in survival, body weights, and food consumption between control and treatment groups were not statistically ($P < 0.05$) significant, either as shown with data or as stated by the authors without data. Median survival was ≥ 73 weeks for all groups in the two studies on ICI mice alone; by terminal sacrifice in the study on four strains (survival patterns were not reported), 52 to 79 percent of the C57BL and CBA mice and 12 to 31 percent of the CF/1 and ICI mice were alive. Liver and kidney weights were slightly lower (data not reported) in male ICI mice given chloroform in toothpaste. The incidences of tumors and lesions between control and chloroform-treated animals that were significantly ($P < 0.05$) different included 1) increased kidney tumor incidences in treated male ICI mice, as shown in Table 8-5, and 2) a significantly ($P < 0.001$, chi-square test) higher incidence of moderate to severe kidney "changes" in treated CBA and CF/1 males than in corresponding controls and of moderate to severe kidney disease ($P < 0.05$, chi-square test) in ICI males given $CHCl_3$ in arachis oil than in arachis oil controls, as described by the authors without presentation of data. Results in Table 8-5 indicate more effective induction of kidney tumors by chloroform in arachis oil than by chloroform in toothpaste. Kidney tumors were not found in C57BL, CBA and female ICI mice, and malignant kidney tumors were diagnosed in two control and one treated

TABLE 8-5. KIDNEY TUMOR INCIDENCE IN MALE ICI MICE TREATED WITH CHLOROFORM (Adapted from Roe et al., 1979)

Dose Group	Numbers of mice examined histologically	Number of mice with kidney tumors		
		Benign	Malignant	Total
First study				
Vehicle-control[a]	72	0	0	0
17 mg $CHCl_3$/kg/day[b]	37	0	0	0
60 mg $CHCl_3$/kg/day[b]	38	5[h]	3[i]	8[j]
Second study				
Untreated control	45	1	0	1
Vehicle-control[a]	237	6	0	6
60 mg $CHCl_3$/kg/day[c]	49	7[h]	2[i]	9[j]
Third study				
Untreated control	83	0	0	0
Vehicle-control[d]	49	1	0	1
60 mg $CHCl_3$/kg/day[e]	47	2	3	5
Vehicle-control	50	1	0	0
60 mg $CHCl_3$/kg/day[g]	48	3	9[h]	12[j]

[a] Toothpaste base vehicle without chloroform, eucalyptol, and peppermint oil.

[b] Chloroform given in toothpaste base with eucalyptol and peppermint oil.

[c] Chloroform given in toothpaste base without eucalyptol and peppermint oil.

[d] Toothpaste base vehicle without chloroform.

[e] Chloroform given in toothpaste base.

[f] Arachis oil.

[g] Chloroform given in arachis oil.

[h] Statistically significant versus vehicle-control ($P < 0.05$).

[i] Statistically significant versus vehicle-control ($P < 0.01$).

[j] Statistically significant versus vehicle-control ($P < 0.001$).

CF/1 mice. Malignant kidney tumors were identified as hypernephromas, and benign kidney tumors were characterized as cortical adenomas. The increased incidences of both the malignant tumors and the benign tumors were each separartely statistically significant, as well as when they were combined.

Eucalyptol and peppermint oil were not toxic to male ICI mice in these studies.

Results of the studies by Roe et al. (1979) show the ability of chloroform to induce kidney tumors in male ICI mice. The stronger induction of kidney tumors by chloroform in arachis oil compared with chloroform in toothpaste may reflect an effect of the dosing vehicle on chloroform absorption and resulting peak blood and tissue levels. Moore et al. (1982) demonstrated greater severity of acute toxicity and regenerative changes in kidneys of male CFLP mice given single gavage doses of 60 mg $CHCl_3$/kg when corn oil rather than toothpaste was the dosing vehicle. Kidney pathology was noted in treated animals in the study with four strains of mice; however, although poorer body weight gain reported for treated mice in each of the strains would suggest that a maximum tolerated dose was being approached, the observation that survival, body weights, and other pathological changes between control and treated mice in each of the four strains were not significantly ($P < 0.05$) different also suggests that higher doses could have been administered to more strongly challenge the mice for carcinogenicity. Since mice were as old as 10 weeks at the start of the studies, it is evident that treatment could have been started when the mice were younger to cover a greater portion of their lifespan during growth. A fuller evaluation of chloroform carcinogenicity could have been made if female mice had also been included in each study.

8.1.2.3. Eschenbrenner and Miller (1945)--An early study on chloroform hepatoma induction in mice was described by Eschenbrenner and Miller (1945). Strain A mice, initially 3 months old, with a notably low historical spontaneous hepatoma rate of <1 percent at 16 months of age were selected for treatment. "Chemically pure" chloroform was used, but a chemical analysis was not indicated in the report. Test groups of five males and five females each were treated by gavage with doses of 2.4, 1.2, 0.6, 0.3, or 0.15 g/kg of chloroform in olive oil. Controls received olive oil alone.

In the study of hepatoma induction, mice were dosed once every 4 days for a total of 30 doses. When 8 months old, the mice were examined for hepatomas at one month after the last dose; however, these animals were given an additional dose of chloroform 24 hours before necropsy. Tissues and organs were examined histopathologically. Liver necrosis also was found in mice given a single gavage treatment of one of the indicated doses of chloroform (one male and two females per group) 24 hours before removal of liver for microscopic evaluation.

Incidences of liver and kidney necrosis and hepatomas are shown in Table 8-6. Liver necrosis was noted in both sexes in the three highest dose groups. Males in all treatment groups developed kidney necrosis, whereas kidney necrosis was not perceptible in females. No males in the three highest-dose groups and no females in the highest-dose group survived the study. All deaths occurred by 48 hours after the second administration of chloroform. All surviving females dosed with 0.6 or 1.2 g $CHCl_3$/kg had hepatomas.

In the experiment to test the ability of a single dose of chloroform to produce tissue necrosis, there was sharp distinction between normal and necrotic cells in liver. Doses of 2.4 and 1.2 g/kg produced extensive necrosis in all liver lobules, and the 0.6 g/kg dose produced necrosis in some lobes. Mice given 30 doses of chloroform in the hepatoma study had moderate liver cirrhosis and necrosis; however, animals given 30 doses that did not result in necrosis had livers that appeared normal. Necrosis was not found in hepatoma cells, and hepatomas contained cords of enlarged liver-like

TABLE 8-6. LIVER AND KIDNEY NECROSIS AND HEPATOMAS IN STRAIN A MICE FOLLOWING REPEATED ORAL ADMINISTRATION OF CHLOROFORM IN OLIVE OIL (adapted from Eschenbrenner and Miller, 1945)

		Dose (g/kg)					
Observation	Sex	2.4	1.2	0.6	0.3	0.15	Control
Liver necrosis	F	+	+	+	0	0	0
	M	+	+	+	0	0	0
Kidney necrosis	F	0	0	0	0	0	0
	M	+	+	+	+	+	0
Deaths[a]	F	5/5	1/5	2/5	0/5	0/5	0/5
	M	5/5	5/5	5/5	2/5	0/5	0/5
Hepatomas in surviving animals receiving 30 doses[a]	F	--	4/4	3/3	0/5	0/5	0/5
	M	--	--	--	0/3	0/5	0/5

[a] Numerator is positive occurrences. Denominator is animals observed.

cells which formed disorganized anastamosing columns. The hepatomas did not appear invasive, and metastasis was not found.

Renal necrosis in males was localized in the areas of the proximal and distal tubules. Glomeruli and collecting tubules appeared normal. The severity of renal necrosis was less with lower doses. The different kidney responses by males and females to chloroform treatment may be due to the unique lining of the Bowman's capsules with flat and cuboidal epithelium in females and males, respectively (an anatomic sexual dimorphism in mice). Although few animals were available for pathologic examination, the Eschenbrenner and Miller study (1945) indicates that hepatomas in female mice were induced at chloroform doses that also produced liver necrosis. Early mortality precluded the development of hepatomas in all animals given chloroform doses that produced liver necrosis. Hepatomas were not induced by non-necrotizing doses of chloroform; however, this was not lifetime study and a lifetime study perhaps could have given a stronger indication of the carcinogenic potential of chloroform at these lower doses. The observation of kidney necrosis in males without tumor formation, and lack of necrosis in hepatomas, suggests that liver in strain A mice was uniquely sensitive to tumor induction at necrotizing doses, or that there might have been additional factors in liver tumor formation besides necrosis. Furthermore, since a dose of chloroform was given 1 day before sacrifice--a factor which in itself could have been responsible for producing necrosis, as supported by liver necrosis found in mice which died after one or two treatments with chloroform--it is not clear what the extent of necrosis was during the last month of observation, when the animals were untreated.

8.1.2.4. Rudali (1967)--Rudali (1967) reported a carcinogenicity study of chloroform in NLC mice. Details such as age and sex of the mice were not given. The mice received twice-weekly doses of 0.1 ml of a 40 percent solution of chloroform in oil by force-feeding for an unspecified treatment period. Twenty-four animals were initially on study, but only five "sound mice" were evidently given a pathologic examination. An average survival period of 297 days was reported, but it is not clear if this period applied to the total group of 24 or to the smaller group of five. An observation period for the study was not mentioned in the report. The use of a control group was not indicated, nor was a chemical analysis of the chloroform sample provided.

Three of the five mice examined in pathology were diagnosed with hepatomas and hepatic lesions; however, details of the pathologic observations were not reported. The study by Rudali (1967) gives evidence for carcinogenic activity by chloroform in NLC mice, even though it is weakened by a lack of experimental details, the absence of a control group, and the small number of animals examined in pathology.

8.1.3. Oral Administration (Drinking Water): Rat and Mouse

8.1.3.1. Jorgenson et al. (1985)--In order to further investigate the reported positive response of experimental animals to chloroform, to validate the studies performed by NCI, and to explore the carcinogenicity of chloroform in drinking water, the route of exposure likely to be encountered by humans, an investigation of the carcinogenicity of chloroform administered in the drinking water of the strains and sexes of mice and rats showing a positive response in the NCI study was undertaken at SRI International (Jorgenson et al., 1985). In these studies chloroform was administered in the drinking water of male Osborne-Mendel rats and female B6C3F1 mice at concentrations of 0 (control), 200, 400, 900, and 1800 mg/L. A second control group was included in the study with water intake restricted to equal that of the high dose group. The animals, both rats and mice, were treated for 104 weeks. The group sizes were larger in the lower level treatment groups so as to increase the likelihood of detecting a carcinogenic response.

Because of questions arising from the conduct of previous studies, the chloroform used in this study was redistilled to minimize the diethylcarbonate, a contaminate noted in the previous study, and the chloroform concentrations in the animal room and feed were monitored. In addition, the blood concentrations of chloroform in the rats were also monitored.

Water consumption was measured during the study. The water intake of the rats was decreased as the chloroform concentration of the drinking water increased. The water intake of the mice was minimally affected after the initial week in which some animals refused to drink. The authors calculated the chloroform daily doses in terms of milligrams per kilogram of body weight, based on the water consumption and concentrations of chloroform in the water. These are tabulated below.

mg/L in water	Rat, mg/kg	Mouse, mg/kg
200	19	34
400	38	65
900	81	130
1800	160	263

In Table 8-7, the tumors that were statistically increased among the rats have been tabulated. The increase in renal tumors among these rats supports the previous finding from the NCI study. The nontumor renal pathology, according to the authors, was high in all groups (91-100 percent). Therefore, it was not possible to relate tumor pathology with nonneoplastic lesions.

Among the female mice, tumor incidence was not increased. In particular, the liver tumors (Table 8-8) were not increased, as had been reported in previous investigations. The high dose in this study, when expressed in terms of unit per kilogram of body weight, was 263 mg/kg/d and therefore essentially the same as the lower dose given by gavage in the NCI study. That similar time-weighted average dose in the NCI study produced an 80 percent response with regard to hepatocellular carcinomas.

It is probable, however, that the peak blood concentrations following a single daily dose by oral gavage in either water or corn oil as the carrier vehicle far exceed those following administration in the drinking water, which results in several intermittent doses during a 24-hr dosing period. As

TABLE 8-7. RELATIVE TUMOR INCIDENCE IN MALE OSBORNE-MENDEL RATS TREATED WITH CHLOROFORM IN DRINKING WATER (Jorgenson et al., 1985)

Tumor Type	Control	Matched Control	Chloroform Concentration (mg/L)				Overall p-value
			200	400	900	1800	
All tumors	212/303[a] (70)[b]	39/50 (78)	227/316 (72)	105/148 (71)	38/48 (79)	34/50 (68)	0.0778[c]
Neurofibroma	2/303 (1)	1/50 (2)	2/316 (1)	1/148 (1)	0/48 (0)	3/50 (6)	0.0167
All lymphomas and leukemias	5/303 (2)	1/50 (2)	19/316[d] (6)	5/148 (3)	2/48 (4)	3/50[d] (6)	0.0368
All circulatory system tumors	5/303 (2)	0/50 (0)	6/316 (2)	3/148 (2)	3/48[d] (6)	3/50[e] (6)	0.0150
All kidney tumors	5/301 (2)	1/50 (2)	6/313 (2)	7/148 (5)	3/48 (6)	7/50[d] (14)	<0.0001
Tubular cell adenoma	4/301 (1)	0/50 (0)	2/313 (1)	3/148 (2)	2/48 (4)	5/50[d] (10)	<0.0001
Tubular cell adenoma and adenocarcinoma	4/301 (1)	1/50 (2)	4/313 (1)	4/148 (3)	3/48 (6)	7/50[d] (14)	<0.0001
Adrenal cortical adenoma	91/298 (31)	16/50 (32)	86/311[e] (25)	36/144[e] (25)	17/48 (35)	11/50[e] (22)	0.0085
Adrenal pheochromocytoma	76/298 (26)	8/50 (16)	71/311 (23)	25/144 (17)	12/48 (25)	5/50[d] (10)	0.0012
Thyroid c-cell adenoma	44/294 (15)	9/49 (18)	33/303[e] (11)	18/148 (12)	6/48[e] (13)	3/50[d] (6)	0.0233
Thyroid c-cell adenomas and carcinomas	47/294 (16)	12/49 (24)	49/303 (16)	27/148 (18)	7/48 (15)	7/50 (14)	0.0335

[a] Number of animals bearing indicated tumors/effective number of animals at risk (continuity corrected).
[b] Figure in parentheses represents percent of animals with the indicated tumor.
[c] Overall p-value calculated using the Peto trend test, for continuity corrected and survival.
[d] Individual treatment group statistically different from control group at $p < 0.01$.
[e] Individual treatment group statistically different from control group at $p < 0.05$.

TABLE 8-8. LIVER TUMOR INCIDENCE RATES IN FEMALE B6C3F1 MICE TREATED WITH CHLOROFORM IN DRINKING WATER (Jorgenson et al., 1985)

Tumor Type	Control	Matched Control	Chloroform Concentration (mg/L)			
			200	400	900	1800
All tumors	225/423[a] (53)[b]	22/47 (47)	217/415 (52)	90/142 (63)	16/47 (34)	24/44 (55)
Hepatocellular adenoma	19/415 (5)	0/47 (0)	8/410 (2)	8/142 (6)	0/47 (0)	0/44 (0)
Hepatocellular carcinoma	2/415 (1)	0/47 (0)	7/410 (2)	1/142 (1)	0/47 (0)	1/44 (2)
Hepatocellular adenoma and carcinoma	21/415 (5)	0/47 (0)	15/410 (4)	9/142 (6)	0/47 (0)	1/44 (2)

[a] Number of animals bearing indicated tumor/effective number of animals at risk (continuity corrected).

[b] Figure in () represents the percent of animals bearing the indicated tumor.

indicated in Chapter 4, Withey et al. (1983) have shown that the postabsorptive blood peak after 75 mg/kg of chloroform by gavage in water was 39 µg/ml and 6 µg/ml in corn oil. By comparison, Jorgenson et al. (1985) indicated for 81 mg/kg (900 mg/L in drinking water) the mean blood concentrations averaged only 75-81 µg/ml. These authors also indicate that they felt the time of sampling (morning) was representative of a blood chloroform concentration following a time when rats were actively drinking. Thus the differences in absorption patterns depending upon the carrier vehicle, the dosing regimen, and the resultant peak blood and tissue levels may help to explain differences in outcomes of the carcinogenicity studies. It has been postulated, but not shown, that corn oil itself may interact in some way to promote the liver tumors in mice. However, the kidney tumors in rats occur regardless of the carrier vehicle or dosing regimen.

8.1.4. Oral Administration (Capsules): Dog

8.1.4.1. Heywood et al. (1979)--The carcinogenicity of chloroform in toothpaste was evaluated in beagle dogs by Heywood et al. (1979). The toothpaste formulation used was that previously described in Table 8-3 except for reduced amounts of carragheen gum and glycerol. Chloroform in toothpaste was transferred from a syringe to gelatin capsules immediately before dosing.

Doses were selected from results of a preliminary range-finding study in which one or two dogs of each sex per group were given oral chloroform doses 7 days per week for 13 (30 and 45 mg/kg/day), 18 (60 mg/kg/day), or 12 (90 and 120 mg/kg/day) weeks. Because 45 mg/kg/day (lowest toxic dose) produced pathologic changes in the liver, dose levels of 0, 15, and 30 mg $CHCl_3$/kg/day were chosen for the carcinogenicity study.

In the carcinogenicity study, chloroform was given orally in capsules 6 days per week for over 7 years. Eight males and 8 females were assigned to each treatment group and to an untreated control group, and 16 dogs of each sex composed a vehicle-control group. The dogs were initially 18 to 24 weeks old. All of the dogs were clinically examined before treatment, and had been receiving medication annually for common diseases. Dogs were fed 200 g of diet twice daily until week 300, when obese dogs received reduced daily rations of 300 g. Body weights, food consumption, and water intake were estimated during the study. Hematology, serum biochemistry, and urinalysis were included in the evaluation of chloroform toxicity. Treatment was stopped at 376 weeks, and survivors were sacrificed for macroscopic examination at 395 to 399 weeks. Major organs were weighed. Tumors, lesions, and routine tissues and organs were evaluated microscopically. Liver and kidney specimens from control and high-dose dogs were also examined by electron microscopy.

Survival, body weights, food and water consumption, and appearance of the eyes were unaffected by chloroform treatment. Mean body weights increased from 7 to 8 kg initially to a maximum of 14 to 15 kg; however, reduction of diet portions for obese dogs complicated the body weight results. Results of blood and urine analyses were unremarkable except for dose-related increases in SGPT levels (Table 8-9), which could reflect liver pathology.

No treatment-related carcinogenic effects were found in necropsy and microscopic examination of tissues and organs. Nonneoplastic diagnoses showed that fatty cysts in the livers of all groups were larger and more numerous in treated dogs.

TABLE 8-9. SGPT[a] CHANGES IN BEAGLE DOGS TREATED WITH CHLOROFORM (adapted from Heywood et al. 1979)

Treatment (mg $CHCl_3$/kg/day)	Group mean SGPT (MU/ml)											
	Pretreatment	Treatment stage (weeks)									Post-treatment (weeks)	
		6	26	52	104	156	208	260	312	372	14	19
30 mg	24	34[b]	58[c]	52[c]	64[c]	76[c]	91[c]	147[c]	128[c]	102[c]	105[d]	111
15 mg	22	29	33	32	45	46[d]	55[d]	95[c]	89[c]	66	53	48
Vehicle-control	22	29	30	29	40	30	40	33	47	51	56	128
Untreated	24	30	30	27	37	29	30	32	50	50	53	56

[a] Serum glutamic-pyruvic transaminase.

[b] Comparison with untreated group; $P < 0.05$.

[c] Comparison with untreated group; $P < 0.01$.

[d] Comparison with untreated group; $P < 0.001$.

The study by Heywood et al. (1979) did not show a statistically significant carcinogenic effect of chloroform in toothpaste given to beagle dogs, although an increased incidence of total number of neoplasms was observed in treated dogs. Range-finding tests and SGPT and liver fatty cyst diagnoses in the carcinogenicity study suggest that a maximally tolerated dose was approached in the carcinogenicity study. It is not certain if 7 years was long enough for carcinogenicity testing with respect to the lifespan of the beagle dog (13 to 14 years), but by 7 years spontaneous tumor formation was becoming evident.

8.1.5. Intraperitoneal Administration: Mouse

8.1.5.1. Roe et al. (1968)--Roe et al. (1968) investigated the carcinogenicity of chloroform in newborn (C57 x DBA2-F1) mice. Chloroform was subcutaneously injected into mice of one group as a single 200 µg dose when the animals were less than 24 hr old, and into mice of another group as eight daily doses of 200 µg each, beginning when the animals were 1 day old. Control groups were given the dosing vehicle, arachis oil, alone. Survivors were sacrificed for necropsy at 77 to 80 weeks.

No carcinogenic effect of chloroform was found. However, since the study was reported as an abstract, experimental details were not provided. Chloroform doses were relatively rather low, and the use of newborn mice given one or a few doses of chloroform is not equivalent to lifetime treatment of animals given doses as high as those maximally tolerated. Additionally, there may be differences in chloroform metabolism between newborn and adult (C57 x DBA2-F1) mice. Hence, it is concluded that this study by Roe et al. (1968) does not present sufficient evidence for an absence of carcinogenic activity by chloroform.

8.1.5.2. Theiss et al. (1977)--The carcinogenicity of chloroform was evaluated by Theiss et al. (1977) by means of the pulmonary tumor induction bioassay in strain A mice.

Test animals were male strain A/St mice initially 6 to 8 weeks old. Preliminary toxicity tests were performed for selection of maximum tolerated doses; in these tests, mice received six intraperitoneal injections of chloroform for 2 weeks and were observed for another 4 weeks. Results of the preliminary tests were not reported. In the bioassay, chloroform doses in tricaprylin were 80 or 200 mg/kg, administered 3 times weekly for a total of 24 intraperitoneal injections; or 400 mg/kg, which was injected only twice. Fifty control mice were given tricaprylin alone. Each treatment group contained 20 animals. Mice were sacrificed 24 hr after the last dose was administered, and the lungs were removed for counting and examining surface adenomas microscopically. The chloroform product given to the mice in this study was reagent grade (Aldrich Chemical Company), but its chemical composition was not reported. A positive control group of 20 mice was given one injection of 1 g/kg of urethane in saline, and compared with 50 controls given saline alone.

Chloroform treatment did not produce a pulmonary adenoma response in this study. The average number of lung tumors per mouse was 0 to 0.39 in each group, except for the positive controls, which had an average of 19.6 lung tumors. At least 90 percent of the mice in each group survived, except for the mice given 400 mg $CHCl_3$/kg, where there was only 45 percent survival. However, since this type of bioassay is basically a screen for carcinogen potential, a negative result does not necessarily indicate a lack of carcinogenic activity. Evidence for the carcinogenic activity of chloroform is available in other studies described in this document, and according to the authors, there is evidence for carcinogenic activity in other compounds, e.g., 2-chloroethyl ether and hexachlorocyclohexane in liver, which also tested negative in the Theiss et al. (1977) study. Carcinogenic effects of chloroform have been shown in the liver and kidney, whereas the lung was apparently not a target organ in the Theiss et al. (1977) study or other chloroform cancer bioassay studies.

8.1.6. Evaluation of Chloroform Carcinogenicity by Reuber (1979)

Reuber (1979) evaluated the carcinogenicity of chloroform based on his review of slides in the NCI (1976) bioassay, and his review of data in other carcinogenicity studies described in this document. Reuber concurred with reported findings of rat kidney tumors and mouse hepatocellular carcinomas in the NCI (1976) study, mouse hepatomas in the Eschenbrenner and Miller (1945) and Rudali (1967) studies, and mouse kidney tumors in the Roe et al. (1979) study. However, Reuber concluded that there was an increased incidence of neoplasms in treated dogs in the Heywood et al. 1979 study. Reuber also concluded that there were treatment-related neoplasms in the NCI study in addition to those reported. In rats, Reuber concluded that chloroform treatment induced liver tumors (hepatocellular carcinomas and neoplastic nodules) and cholangiofibromas and cholangiocarcinomas in addition to kidney tumors. Besides hepatocellular carcinomas, Reuber concluded that malignant lymphoma was also induced by the chloroform treatment in mice. Reuber noted that treated rats and mice did not exhibit liver cirrhosis, that treated rats with thyroid tumors generally did not have liver or kidney tumors, and that liver necrosis was apparent only in high-dose female mice. The differences in histopathologic interpretation of tissue specimens in the NCI bioassay between the Reuber study and the NCI report, outside of a difference of opinion between pathologists, are not clear.

8.1.7. Oral Administration (Drinking Water): Mouse: Promotion of Experimental Tumors

8.1.7.1. Capel et al. (1979)--The effect of chloroform ingestion on the growth of murine tumors was assessed by Capel et al. (1979). Redistilled analar chloroform was used, but chemical analysis of the product was not indicated. Test animals were male C57CL/105cSn/Ola and male Theiller-Original (TO) mice, 20 to 22 g body weight. A cage of 20 mice drank 80 to 100 ml of water each day; hence, chloroform was added to yield estimated doses of 0.15 or 15 mg $CHCl_3$/kg/day for two dose groups, with each mouse drinking 4 ml water per day. Fresh chloroform solution was given daily and was protected from light.

In one experiment, 100 TO mice in each dose group were divided into three approximately equal subgroups. One subgroup (pretreated) was treated with chloroform for 14 days before and after inoculation of Ehrlich ascites tumor cells. Another subgroup (post-treated) was given chloroform only after inoculation of tumor cells. The third subgroup, also inoculated with tumor cells, served as untreated controls. Tumor cells had been maintained in the peritoneal cavity of male TO mice by weekly passage of 10^6 cells. Peritoneal fluid was collected 7 days after inoculation of cells and diluted with buffered saline. All mice in the three subgroups were given intraperitoneal injections of 0.1 ml diluent (10^6 cells). At the end of exponential growth at 10 days following inoculation of cells, animals were sacrificed for removal of peritoneal fluid. The peritoneal cavity was washed with heparinized buffered saline. Fluid and washings were combined and diluted with buffered saline. Cells were disrupted by sonication for estimation of DNA levels per ml cell suspension as a measure of total cell content.

A second experiment was done in which 100 C57BL mice in each dose group were subdivided into three groups (pretreated, post-treated, and control), each of which was treated with chloroform according to the corresponding protocol in the first experiment. Each mouse received a subcutaneous injection of 10^6 B16 melanoma cells suspended in 0.1 ml buffered saline. Inoculum was obtained from a C57BL mouse that had received a transplant of syngeneic B16 melanoma cells maintained by intramuscular passage every 14 days. Animals were sacrificed at 21 days after inoculation, and spleen, mesenteric lymph nodes, and lungs were examined for metastases.

In the third experiment, Lewis lung tumor cells were maintained by serial intramuscular transplantation in C57BL mice. A group of 100 mice was divided into three approximately equal subgroups (pretreated, post-treated, control) to investigate the effect of 15 mg $CHCl_3$/kg/day on tumor growth and spread according to the protocol used in the first experiment. Each mouse received intramuscular thigh injections of 2 x 10^6 cells suspended in 0.1 ml buffered saline. Animals were killed 14 days after the administration of tumor cells, and both the tumor-bearing and the normal thighs were skinned and severed at the knee and hip. Tumor weight was estimated as the difference between the weights of the thighs. Pulmonary tumor foci were also counted.

For estimation of the effect of 0.15 mg $CHCl_3$/kg/day in the third experiment, 100 mice were divided into subgroups of 20 animals each and were pretreated with chloroform before (for 8, 6, 4, or 2 weeks) and after injection of the Lewis cells. Mice were sacrificed at 16 days after inoculation of tumor cells, and tumor weights and numbers of lung foci were determined. In these animals, homogenates of primary tumors were prepared for β-glucuronidase estimation and protein content.

The results of these experiments are summarized in Tables 8-10, 8-11, and 8-12. Body weights and survival were not affected by chloroform treatment. Ehrlich ascites tumor cells, as equated with DNA content, were significantly ($P < 0.05$) increased in high-dose mice, and slightly, though not significantly, increased in low-dose animals. Invasion by B16 melanoma cells, especially in the spleen, was augmented by both doses, and the numbers of lung foci were also greater in both treatment groups. Metastasis of Lewis cells was increased only by treatment with 15 mg $CHCl_3$/kg. There was no change in β-glucuronidase levels based on tumor protein content in the low-dose group. The increased tumor protein levels appear to reflect tumor growth which was not evident by weighing.

The study by Capel et al. (1979) shows an ability of chloroform to enhance the growth of three types of murine tumors in mice. A dose of 15 mg $CHCl_3$/kg was effective in each experiment, whereas a dose of 0.15 mg $CHCl_3$/kg was effective only in the test with B16 melanoma cells. Although this study does not evaluate the ability of chloroform to induce primary tumors, it does give evidence for a promoting effect of chloroform on the growth and spread of experimental tumors at low doses. However, the mechanism by which chloroform enhanced tumor growth in the study by Capel et al. (1979) is not certain, and the relevance of this study to the overall evaluation of the carcinogenic potential of chloroform is not clear.

TABLE 8-10. EFFECT OF ORAL CHLOROFORM INGESTION ON THE GROWTH OF EHRLICH ASCITE TUMORS
(Capel et al., 1979)

Dose	Treatment group	Number of animals per group	Average body weight (g)[a]	Tumor DNA (μg/ml)[a]	Significance
0.15 mg/kg/day	Control	33	38.3 ± 3.7	661 ± 222	
	Post-treated	33	39.4 ± 2.9	724 ± 254	NS
	Pretreated	33	37.9 ± 3.2	770 ± 283	NS
15 mg/kg/day	Control	43	39.4 ± 3.4	637 ± 221	
	Post-treated	37	37.5 ± 3.0	1143 ± 324	$P < 0.001$
	Pretreated	30	37.0 ± 3.9	827 ± 245	$P < 0.001$

[a] Results expressed are the mean ± S.D.
NS = Not significant; $P > 0.05$.

TABLE 8-11. EFFECT OF ORAL CHLOROFORM INGESTION ON METASTATIC "TUMOR TAKES" WITH B16 MELANOMA (Capel et al., 1979)

Animals with B16 melanoma invasion in organics (%)						
					Lung	
Dose	Treatment	Number of animals per group	Spleen	Mesenteric lymph nodes	(a)[a]	(b)[b]
0.15 mg/kg/day	Control	26	15	13	12	3
	Post-treated	31	35	10	10	5
	Pretreated	28	36	29	18	10
15 mg/kg/day	Control	30	15	12	6	4
	Post-treated	32	31	25	19	6
	Pre-treated	32	31	32	20	20

[a] Numbers in column (a) refer to the percentage of animals with tumor foci on the lungs.
[b] Numbers in column (b) refer to the average number of lung metastases.

TABLE 8-12. EFFECT OF ORAL CHLOROFORM INGESTION ON THE GROWTH AND SPREAD OF THE LEWIS LUNG TUMOR[a]
(Capel et al., 1979)

Dose	Treatment	Number of animals per group	Average body weight (g)	Tumor Weight (g)	Lung metastases	Significance	B-glucuronidase activity[b]	Protein content[c]
0.15 mg/ kg/day	8[d]	20	30.6 ± 3.8	3.5 ± 0.81	165 ± 56	NS	0.33 ± 0.56	78.2 ± 4.2
	6	20	30.6 ± 3.8	3.3 ± 0.72	170 ± 41	NS	0.27 ± 0.79	66.8 ± 2.7
	4	20	29.0 ± 3.6	3.3 ± 0.54	154 ± 39	NS	0.38 ± 0.073	60.1 ± 5.1
	2	20	29.0 ± 3.5	3.2 ± 0.72	147 ± 44	NS	0.49 ± 0.070	60.3 ± 4.7
	0 (control)	20	29.3 ± 2.7	3.1 ± 0.11	142 ± 34		0.58 ± 0.094	50.8 ± 6.2
15 mg/ kg/day	Control	33	23.6 ± 1.3	1.6 ± 0.31	44 ± 26			
	Post-treated	33	24.5 ± 2.3	1.7 ± 0.51	57 ± 19	$P < 0.05$		
	Pretreated	33	24.4 ± 2.2	1.8 ± 0.12	61 ± 19	$P < 0.01$		

[a] Results expressed are the mean ± S.D.
[b] Expressed as mole product/mg protein/min.
[c] Milligrams of protein after extraction mg/g wet weight.
[d] Duration of treatment (weeks).
NS = not significant, $P>0.05$.

8.2. CELL TRANSFORMATION ASSAY

8.2.1. Styles (1979)

Styles (1979) reported an investigation of chloroform in a cell transformation system with BHK cells, using growth in semi-solid agar as an endpoint, as part of a larger study (Purchase et al. 1978) conducted to screen chemicals for carcinogenic potential. The BHK-agar transformation assay technique used has been previously described by Styles (1977) and by Purchase et al. (1978). In the chloroform study reported by Styles (1979), baby Syrian hamster kidney (BHK-21/Cl 13) cells were exposed to five different doses of test substance *in vitro* in serum-free liquid tissue culture medium in the presence of rat liver microsomal fraction and cofactors (S-9 mix; Ames et al., 1975). The liver microsomal fraction was obtained from Sprague-Dawley rats induced with Arochlor 1254.

Cells were grown and maintained in Dulbecco's modification of Eagle's medium in an atmosphere of 20 percent CO_2 in air. Cells were maintained at 37°C until confluent, and then were trypsinized and resuspended in fresh growth medium. Resuspended cells were grown until 90 percent confluent for transformation assays or 100 percent confluent for stock. Only cells with normal morphology were used for assays. To minimize spontaneous transformation frequency, cells were obtained at low passage, grown to 90 percent confluency, and frozen in liquid nitrogen. Cells were thawed at 37°C in growth medium for further use.

Test compounds were dissolved in DMSO or water as appropriate. Each dose was tested in replicate assays. Cells incubated until 90 percent confluency were trypsinized and resuspended in Medium 199 at a concentration of 10^6 cells/ml. Resuspended cells (10^6) were incubated with test chemical and S-9 mix at 37°C for 4 hours. After treatment, cells were centrifuged and resuspended in growth medium containing 0.3 percent agar. Survival after treatment was estimated by incubating 1,000 cells at 37°C for 6 to 8 days before counting colonies. Transformation was evaluated by counting colonies after cells were plated and incubated for 21 days at 37°C. The dose-response for transformation was compared with that for survival. Styles (1977) accepted a fivefold increase in transformation frequency above control values at the LC_{50} as a positive result. The spontaneous transformation frequency of BHK cells (72 experiments) in this study was 50 ± 16 per 10^6 survivors. The suitability of the soft agar medium for colony growth was checked by assays with polyoma-transformed BHK-21/Cl 13 cells or Hela cells.

Cell transformation results were negative with exposure to chloroform solution in DMSO added to culture medium in a dose range that included levels at which toxicity was observed (Figure 8-2). Although chloroform doses high enough to produce toxicity did not induce transformation, exposure of cells to chloroform as a vapor could have provided a comparison of the transformation potential of chloroform as a vapor and chloroform in liquid solution.

The study by Purchase et al. (1978), which was done on 120 chemicals of various classes, showed that the BHK-agar transformation assay system was about 90 percent accurate in discriminating between compounds with demonstrated carcinogenic or noncarcinogenic activity, and was in approximately 83 percent agreement with the results of assays done by the authors with *S. typhimurium* (TA 1535, TA 1538, TA 98, TA 100). Styles (1979) indicated, without presenting numerical data, that the results obtained in *Salmonella* assays on chloroform in liquid solution were similar to the findings of the transformation assays. Purchase et al. (1978) also observed that metabolically activated agents transformed BHK cells more

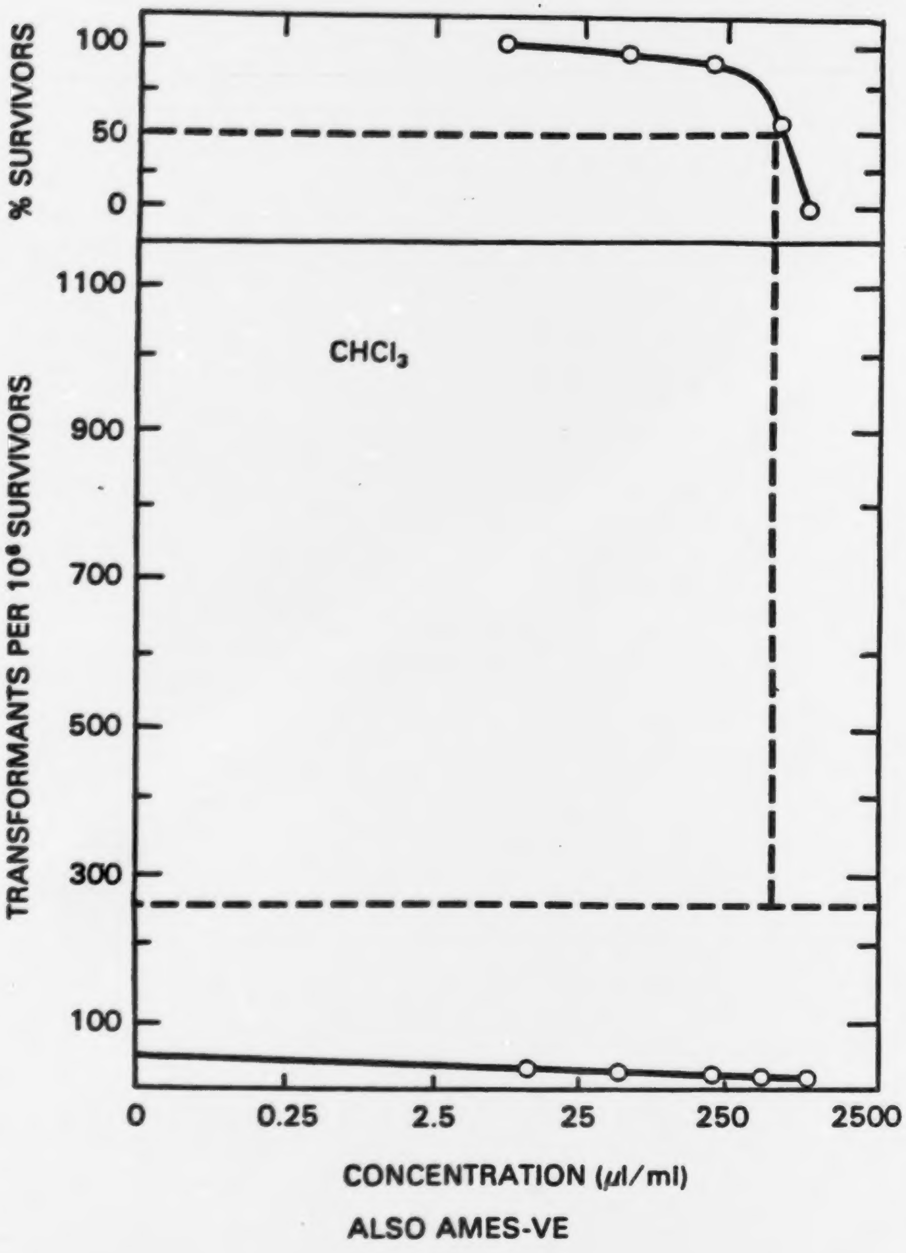

Figure 8-2. Negative result in transformation assay of chloroform, which was also negative in the Ames assay.

Source: Styles (1979).

strongly in the presence of S-9 mix, thus suggesting that BHK cells have limited intrinsic metabolic capability.

8.3. EPIDEMIOLOGIC STUDIES

In the last decade there has appeared in the literature a host of epidemiologic and statistical studies of cancer and exposure to the constituents of drinking water, of which chloroform is one (Harris, 1974; Page et al., 1976; Tarone and Gart, 1975; Buncher, 1975; Vasilenko and Magno, 1975; De Rouen and Diem, 1975; McCabe, 1975; Kruse, 1977; Alavanja et al., 1978; Rafferty, 1979; Kuzma et al., 1977; Harris et al., 1977; Salg, 1977; Mah et al., 1977; Brenniman et al., 1978; Tuthill et al., 1979; Wilkins, 1978). These studies have been subjected to several critical reviews (Wilkins et al., 1979; U.S. Environmental Protection Agency, 1979; National Academy of Sciences, 1978) and have been discussed in some detail. Some very general relationships have been noted by the reviewers. Of particular importance is the appearance of some consistency in the finding of cancer of the large intestine, rectum, and bladder associated with the constituents of drinking water.

It must be emphasized that none of the studies discussed in this section implicates chloroform directly as the sole or dominant constituent of drinking water responsible for the excess of cancer at these sites. Over 300 volatile organic contaminants have been identified in drinking water, and many of these have been identified as carcinogens (Wilkins et al., 1979).

However, chloroform at a peak concentration of 266 μg/L has been shown to exceed peak concentrations of other detected carcinogens by levels 37 times higher than those of the next highest carcinogen, vinyl chloride (Wilkins et al., 1979).

Chloroform measurements appear to range largely between 1 and 112 µg/L, according to a survey of 76 drinking water supplies (Cantor et al., 1978).

Although a direct association cannot be made, the possibility still exists that since chloroform is apparently the predominant component in chlorinated drinking water, it could be a contributing factor in the etiology of the cancer associated with the consumption of drinking water.

Almost all of the above-referenced studies were ecological correlation investigations, and only a few utilized case-control methods. The studies varied by sample size, cancer sites considered, control variables, and the types of end points used as indicators. Among the problems posed by the data in these studies are the following: 1) a lack of data measuring the quantity of chlorine and chloroform in drinking water; 2) the limited nature of recently acquired data on the quality and quantity of organics in drinking water; 3) the limited amount of information given regarding personal consumption of drinking water; 4) the long latency periods associated with most cancers (current cancer rates reflect exposures received decades earlier); and 5) the demographic effects of migration, which adds another dimension of difficulty to the quantification of personal consumption of drinking water over time.

Since publication of the three reviews referred to above, several additional studies of cancer and exposure to trihalomethanes have been published. The following pages discuss each of these studies in detail.

8.3.1. Young et al. (1981)

Young et al. (1981) conducted a case-control study in which cancer deaths in 8,029 white females were matched with non-cancer deaths in some 8,029 white females for county of residence, year of death, and age recorded on death certificates in 28 counties in the State of Wisconsin from 1972 through 1977. Information about the chlorine content of the drinking water of the 16,058 cases and controls was derived from mail-back questionnaires recently submitted to the superintendents of 202 waterworks encompassing the counties sampled. The questions pertained to prechlorination and postchlorination dosages used over the past 20 years (average daily dose in ppm). For 14% of the sample who were not served by a waterworks, decedents were assigned chlorine dosages of zero. The assignment was on the basis of water supplied to decedent's usual place of residence.

Odds ratios were calculated from a logistic regression model. This model provided estimates of the relative risk of site-specific cancer deaths for exposure of the previous 20 years to high, medium, and low chlorine doses, as compared with no chlorination. Urbanicity, marital status, and site-specific high-risk occupation were controlled in the model. Only colon cancer showed a significant ($P < 0.05$) association with chlorine intake in all three dosage categories. However, no gradient of increasing risk with increasing dosage was apparent. For the high, medium, and low dosage categories, the odds ratios were 1.51, 1.53, and 1.53, respectively. All were significant at $P < 0.05$. In those counties where the drinking water supplies were exposed to rural runoff, the odds ratios for colon cancer increased to 3.43, 3.68, and 2.94 for high, medium, and low average daily chlorine doses when controlled for water source depth and purification. These were statistically significant at the $P = 0.025$ level. Colon cancer mortality was not related to chlorination in counties not exposed to rural runoff. This finding is consistent with the hypothesis that trihalomethanes are formed through the action of chlorine on organic substances in drinking water.

Nonsignificant risks were evident at the remaining sites, i.e., esophagus, stomach, rectum, liver, pancreas, kidney, bladder, lung, brain, and breast. The average daily chlorine dose categories were designated by the authors as follows: none (less than 0.01 ppm), low (0.01-0.99 ppm), medium (1.00-1.70 ppm), and high (1.71-7.00 ppm).

The authors made a number of assumptions regarding exposure of subjects and controls to chloroform. They assumed that chlorine in drinking water would represent a good surrogate for exposure of cases and controls to chloroform, reasoning that trihalomethanes such as chloroform are believed to result from the reaction of chlorine with naturally occurring organics in water. Although drinking water at the tap was not analyzed for chloroform or other trihalomethanes, the authors assumed that the measured levels of chlorine at the respective waterworks would correlate well with presumed exposure to chloroform in drinking water.

Such implicit assumptions appear questionable for several reasons. First, the latent period for the development of several, if not most, of the cancer sites is most probably greater than 20 years. This is longer than the period covered by the exposure data on chlorination of water supplies used by the authors.

Second, migration within and around the 28-county area could have masked any real risk that was related to exposure. A diagnosis of colon cancer, which has a 5-year survival rate of better than 46 percent, could have induced victims to migrate to urban areas (where chlorine levels were higher) in order to obtain better medical care, thus leading to a false positive association.

Third, the amount of chloroform that is formed from the addition of chlorine is a function of several important variables: the quantity of organics in the water supply, treatment practices, and chlorine dosages. The quantity of organics in the water supply is, in turn, determined by the nature of the water supply source. Surface water (rivers and streams) receives large quantities of organics from land runoff, whereas groundwater contains little or no organic material; hence, the likelihood of chloroform formation from the addition of chlorine to a groundwater supply is minimal.

Fourth, liquid intake rates and amounts vary considerably from person to person. It is clear that most people satisfy their liquid requirements through a variety of drinks besides tap water, (e.g., milk, orange juice, coffee, soda). It is conceivable that many may drink little water because of these competing sources of liquid refreshment. Therefore, it is probable that many persons who were ranked as having been exposed to chloroform may in fact have had little exposure to it. The resulting misclassification of cases and controls by exposure category would tend to mask any gradient of increasing risk with exposure if one existed.

Another possibly confounding variable not controlled for in this study is the dietary intake of meat and foods low in fiber content (Reddy et al. 1980), both of which have been hypothesized as being related to colon or rectal cancer. The dietary intake of such foods, however, is not known to be correlated with the quantity of chlorine in drinking water, although the possibility of a spurious correlation cannot be ruled out. In more urbanized counties where chlorine levels are higher, residents may consume a diet of more meat and less fiber.

In summary, a definite association of chlorine or chloroform in drinking water with an increased risk of colon cancer should not be made for the reasons stated.

8.3.2. Hogan et al. (1979)

Hogan et al. (1979) conducted an ecological study of site-specific cancer rates based on NCI cancer mortality data by county for the years between 1950 and 1969 (Mason and McKay, 1974) and on chloroform levels in finished drinking water, as determined by the U.S. EPA in two separate surveys (U.S. EPA, 1975). The first survey, known as the National Organics Reconnaissance Survey (NORS), consisted of samples from 80 water treatment facilities across the country. The second survey covered 83 utilities in the states of Illinois, Indiana, Michigan, Minnesota, Ohio, and Wisconsin. Linear multiple regression analyses were done for each set of data separately. The dependent variable was county site-specific cancer mortality. Weighted and unweighted regression coefficients were determined for a number of independent variables selected by the author based on a study by Hoover et al. (1976). A variety of demographic characteristics related to cancer mortality were used in addition to the variable "chloroform levels" as determined from the NORS and regional surveys to explain cancer mortality. These characteristics were as follows: county population density, percent of urbanization per county, percent of nonwhite people, percent of foreign-born, county median family income, educational level, percent of workforce employed in manufacturing, chloroform level in drinking water samples, and county population. According to the authors, the weighting was based on the inverse of the square root of the population of the race-sex county stratum, and was done chiefly to improve the precision of the regression estimates.

Significant positive statistical correlations were found between chloroform levels in treated drinking water and cancer mortality specific for bladder, rectum, and large intestine in the "weighted" regression for white females. On the other hand, only stomach cancer appeared to be positively correlated significantly with chloroform levels in white males. Without weighting, cancers of the bladder, rectum, thyroid, and breasts were significantly correlated with chloroform levels in white females. In white males, cancers of the pancreas and rectum were significantly correlated with chloroform without weighting. Only estimated regression coefficients were provided with their corresponding P values. The study contained no information on actual levels of chloroform observed in drinking water. Nonwhites were not considered because of the small sizes of the populations from which rates were derived.

Ecological studies such as this one are necessarily weak because their information is based on aggregate rather than individual data. The evidence for an association is indirect and definite conclusions cannot be drawn, although hypotheses may be formulated. It is not certain whether a multiple linear regression technique is the proper method for analyzing such data, since the assumption of linearity implied in its selection may not be warranted. Also, since the model contains no interaction terms, it is implicit that the chosen control variables are independent of each other, and such an assumption may also be unwarranted. Furthermore, as was mentioned in the Young et al. (1981) study, these data are weakened because it was assumed that the subjects were actually exposed to the levels of chlorine (or chloroform) indicated. Another limitation is that since the chloroform data were collected in 1975, the more relevant exposure data (assuming a general cancer latency of 10 to 30 years) should be those of 1920 to 1959, given that the site-specific cancer mortality data covered the period 1950-1969.

8.3.3. Cantor et al. (1978)

Cantor et al. (1978), in an ecological study of cancer mortality and halomethanes in drinking water, used age-standardized cancer mortality rates by site and sex in whites for the years 1968-1971, but only in the 923 U.S. counties that were more than 50% urban in 1970. This study was similar to the Hogan et al. (1979) study with respect to its design; i.e., a weighted linear regression model was used with sex- and site-specific cancer rates as the dependent variable. The weight was directly proportional to the square root of the counties' person-years at risk and thus inversely proportional to the standard deviation of the estimated mortality rate. Chloroform ($CHCl_3$), bromochloromethane (BTHM), and total trihalomethane (THM) levels were obtained from the two EPA surveys (U.S. EPA 1975) used in the Hogan et al. study. Demographic variables used in the regression model on a county-wide basis were as follows: percent of urbanization (1970); median school years completed by persons over age 25; population size (ratio of 1970 to 1950 population); percentage of the work force in manufacturing; and percentage of foreign-born. Although a predicted, age-adjusted, site-specific cancer rate was calculated for each county based on this regression technique, only the data for 76 counties, where more than half of the population of the counties was served by a sampled water supply, were actually used in this correlation analysis of THM levels with residual mortality rates. Figure 8-3 gives a frequency distribution of the chloroform levels in these 76 U.S. drinking water supplies. The three indicators, chloroform, bromochloromethane, and total trihalomethane, were highly correlated with one another.

Positive nonsignificant correlations with THM levels were evident with respect to several forms of cancer, including lymphoma and kidney cancer in males (Table 8-13). But according to the authors, bladder cancer mortality rates gave the strongest and most consistent association with THM exposure after controlling for differences in social class, ethnic group, urbanicity, region, and extent of county industrialization (Table 8-14). However, the

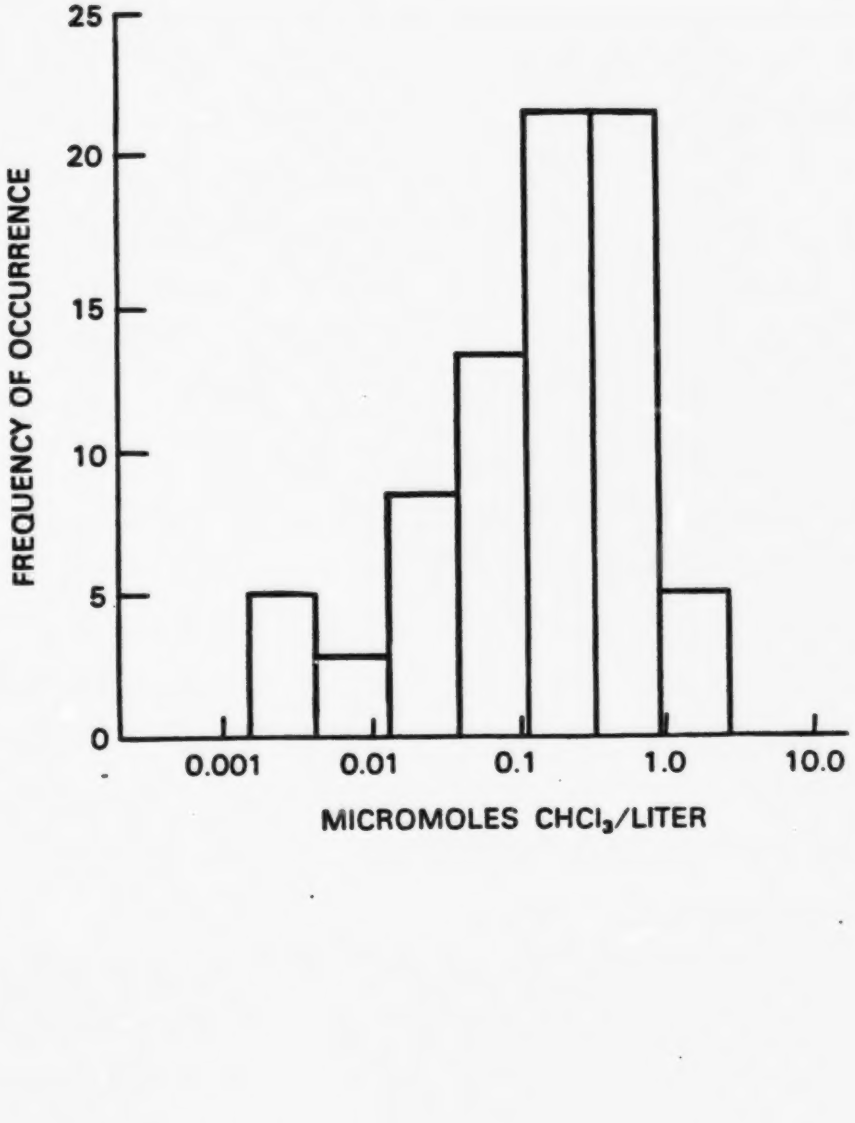

Figure 8-3. Frequency distribution of $CHCl_3$ levels in 76 U.S. drinking water supplies. The abscissa is linear in the logarithm of the level.

Source: Cantor et al. (1978).

TABLE 8-13. CORRELATION COEFFICIENTS BETWEEN RESIDUAL MORTALITY RATES IN WHITE MALES AND THM LEVELS IN DRINKING WATER BY REGION AND BY PERCENT OF THE COUNTY POPULATION SERVED IN THE UNITED STATES (Cantor et al. 1978)

Site of cancer	THM Indicator	Correlation coefficients for regions of the U.S.			
		North	South	Mountain Pacific	All regions
Kidney	$CHCl_3$	0.11 (0.54)[a]	-0.11 (0.73)	0.66 (0.11)	0.14 (0.33)
Lymphoma (non-Hodgkins)	BTHM	0.06 (0.74)	0.08 (0.79)	0.05 (0.92)	0.06 (0.70)
		Correlation coefficients for counties in which the percent of the population served was:			
		50-64%	65-84%	85-100%	50-100%
Kidney	$CHCl_3$	-0.16 (0.44)	-0.11 (0.60)	0.42 (0.04)	0.07 (0.55)
Lymphoma (non-Hodgkins)	BTHM	-0.33 (0.11)	-0.19 (0.36)	0.36 (0.08)	-0.08 (0.81)

[a] P value for two-tailed t-test is shown in parentheses.

TABLE 8-14. CORRELATION COEFFICIENTS BETWEEN BLADDER CANCER MORTALITY RATES BY SEX AND BTHM LEVELS IN DRINKING WATER BY REGION OF THE UNITED STATES (Cantor et al. 1978)

	Correlation coefficients by region			
Bladder cancer	North	South	Mountain	Total
Number	31	13	7	51
Male white	0.52[a] (0.002)	0.04 (0.90)	-0.02 (0.96)	0.30 (0.03)
Female white	0.30 (0.11)	0.20 (0.51)	0.63 (0.13)	0.33 (0.02)

[a] P value for two-tailed t-test is shown in parentheses. Counties with at least 65% of their populations served by one water supply were included in this analysis.

association appeared to be greatest with respect to BTHM and not chloroform. The corresponding correlations for chloroform were positive but nonsignificant. The authors report that although other sites appeared to be positively correlated with THM levels, the inconsistencies "outweigh the consistencies," thus casting doubt on the reliability of these correlation coefficients; i.e., the direction and strength of the correlations bear little relationship to the percent of population served by treated drinking water and/or by region.

The authors noted an association of kidney cancer with chloroform exposure that was restricted to males, but was significant only in counties where at least 85 percent of the public was served by treated drinking water. In counties where less than 85 percent was served by treated drinking water, the correlation coefficients were actually negative. Combining all counties with greater than 50 percent served by treated drinking water, the correlation coefficient was nonsignificant and close to zero. One interesting observation was that without controlling for ethnicity, the authors found a "fairly strong" association of THM levels with colon cancer and lung cancer rates in both sexes, and even a dose-response relationship between these tumor sites and the proportion of the population exposed. However, when ethnicity was added to the regression model, these relationships disappeared.

Again, this is a descriptive study from which hypotheses can be formulated only for future in-depth study. It cannot be concluded that even the significant positive correlations in the study indicate any evidence of real associations. As the authors point out, potential sources of error (i.e., control of confounders such as cigarette smoking and diet) are particularly difficult since no direct information is available on the individuals studied. The main problem with such studies, as mentioned earlier, is that the data are aggregate rather than individual. Such data frequently include large numbers of individuals who never received the exposure in question. Associations derived from such data may be misleading and are often unreliable.

8.3.4. Gottlieb et al. (1981)

Gottlieb et al. (1981) completed a case-control study of the relationship between Mississippi River drinking water and the risk of rectum and colon cancer. The study was based on mortality data gathered from 20 parishes in southern Louisiana. Rectal and colon cancer deaths (692 and 1167, respectively) from 1969 to 1975 were matched one-to-one to noncancer deaths by age at death, year of death, sex, and race, with respect to industrial and urban-rural characteristics, which were defined so that each parish included nearly equal populations using groundwater and surface water supply sources, based on information from the 1970 census.

Three different estimators of exposure were used. The first, "sourcelife," is defined as follows: "mostly surface" (birth and death in a surface-water-using parish); "some surface" (some known surface water use at birth or death); "possible surface" (death in a groundwater parish but had either unknown or out-of-state birthplace); and "least surface" (birth and death in a groundwater-using parish). Length of residence was also considered, if known and for more than 10 years. The second index used was chlorine level (none, low [less than 1.09 ppm], or high [greater than 1.08 ppm]). The third index was the level of organics in the drinking water (low [less than 68 ppm] and high [greater than or equal to 68 ppm]). Sourcelife could be determined for 99.2 percent of the entire group of 3718 cases and controls, but 51 percent had no data for length of residence or had lengths of residence of under 10 years. For those with lengths of residence of less than 10 years, water sources during the possible carcinogenic period were unknown. Chlorine values were available for 78.9 percent of the 3718 sources, while organic levels were available for only 50.1 percent of the sources. The analyses using the latter two variables were equivocal, possibly due to the lack of information on these parameters.

Colon cancer was found not to be related significantly to any water variable, although the number of colon cancer cases in this study (1167) was greater than the number of rectum cancer cases (692). The authors hint that the earlier correlation found in ecological studies could have resulted from confounding with urban lifestyles. Rectal cancer, on the other hand, was found to be significantly elevated with respect to surface or Mississippi River water consumption. Based on sourcelife, the odds ratio for rectal cancer for those who were born and died using groundwater sources was 2.07 (95% confidence interval [C.I.] 1.49-2.88) based on a multidimensional contingency table analysis. Chlorination was significantly associated with rectal cancer, and for those who used river water, the risk decreased as the distance from the mouth of the river increased. The odds ratio for cancer of the rectum at a location below New Orleans versus one above the city was 1.82 (95 percent C.I. 1.01-3.26). The authors noted that both sexes were at increased risk. With respect to controlling for the effect of chlorination where adequate numbers existed, the surface water versus groundwater effect on rectal cancer was of only borderline significance ($P = 0.05$), implying a chlorine effect.

On the other hand, information on organics levels was available for over 48 percent of the rectal cancer group and their controls. The odds ratio calculated based on these data was nonsignificant (Table 8-15), but was probably subject to some bias with respect to availability of exposure data as a function of date of death.

TABLE 8-15. RISK OF MORTALITY FROM CANCER OF THE RECTUM ASSOCIATED WITH LEVELS OF ORGANICS IN DRINKING WATER (Gottlieb et al., 1981)

	Cases	Controls
High (≥ 68 ppm)	110	97
Low (< 68 ppm)	232	220
Total	342	317
Odds ratio	1.08	

With respect to colon cancer, the authors felt that since they had grouped the parishes according to industry and urban characteristics (matching was done within the parish group), they successfully eliminated urban lifestyle as a confounder in their evaluation of colon cancer and drinking water.

The results of this study suggest that cancer of the rectum is linked to the consumption of surface water, and since chlorination appears to be an effect modifier altering the risk ratio to only borderline significance, it would seem that chlorination does contribute to the risk of rectal cancer.

8.3.5. Alavanja et al. (1978)

Alavanja et al. (1978) reported on a case-control study of 3446 gastrointestinal and urinary tract cancer deaths (1595 females and 1851 males) occurring during a three-year period from 1/1/68 to 12/31/70 in seven counties of New York State. Some 3444 individually matched noncancer deaths were also selected. Independent variables were 1) residence in an urban or rural area, 2) residence in an area served by chlorinated or nonchlorinated water, 3) residence in an area served by surface water or groundwater, and 4) occupation. Cases were taken from computer tapes of New York State death certificates and were individually matched with an equal number of non-cancer deaths for the same year. Matching variables were age, race, sex, foreign- versus United States-born, and county of usual residence. If potentially confounding variables could not be controlled via the matching process, the cases and controls were stratified by these confounding variables. The data were analyzed by the chi-square test. A statistically significant excess of gastrointestinal and urinary tract cancer mortality occurred among women in the urban county of Erie (odds ratio [OR] = 2.08), with nonsignificant excesses in Schenectady County (OR = 2.98) and Alleghany County (OR = 4.13). Likewise, among men a statistically significant excess of gastrointestinal and urinary tract cancer mortality occurred in Erie County (OR = 2.15) and Rensselaer County (OR = 1.98), and a nonsignificant excess occurred in Schenectady County (OR = 1.96) and Allegany County (OR = 2.85). Although the study encompassed a seven-county area, almost two-thirds of the deaths occurred in Erie County. The combined overall odds of dying from gastrointestinal and urinary tract cancer for all seven counties combined (including Erie), were only 1.79 based on 3446 cases, whereas in Erie County alone they were 3.15 based on 2177 cases. The authors concluded that males and females residing in the chlorinated water areas of the counties noted above were at a greater risk of gastrointestinal and urinary tract cancer mortality not due to age, race, ethnic distribution, urbanicity, occupation, inorganic carcinogens (Cd, As, Be, Pb, Ni, NO_3), or surface/groundwater difference. No environmental data are provided, however, to characterize quantities of chlorine (or chloroform) exposure. "Inadequate water quality data" prevented the authors from making a "definitive claim that the process of chlorination is directly or indirectly responsible for the greater risk of gastrointestinal and urinary tract cancer mortality" in chlorinated cancer areas. No description is given of how residence was classified into chlorinated versus nonchlorinated water areas or surface water versus groundwater areas through the use of water distribution maps, a practice which can result in misclassification on the basis of exposure. Again, because of the lack of individual dosage data on chloroform exposure and the low significance of the risks described, this study can only be regarded as suggestive for gastrointestinal and urinary tract cancer mortality.

8.3.6. Brenniman et al. (1978)

Brenniman et al. (1978) attempted to confirm the findings of Alavanja et al. (1978) in a case-control study of gastrointestinal and urinary tract cancer mortality among whites in 70 Illinois communities using both chlorinated and nonchlorinated groundwater. The authors limited the study to groundwater because of the possible introduction of confounding effects due to agricultural runoff and industrial sewage in surface water. The 3208 cases and 43,666 controls used were those of Illinois deaths occurring between 1973 and 1976. Controls were selected from a pool of noncancer deaths after the elimination of certain special types of deaths, such as perinatal deaths.

Chlorinated groundwater communities were matched with nonchlorinated groundwater communities that were similar with respect to urbanicity and Standard Metropolitan Statistical Area (SMSA) description. To ensure a minimum follow-up period, water supplies were categorized as chlorinated or nonchlorinated according to a "1963 inventory of municipal water facilities."

Additionally, questionnaires were sent to water treatment plants in the communities to verify the 1963 data. The beginning dates for chlorination were obtained for many of the plants. Based on an EPA survey, it was found that 14 chlorinated groundwater supply sources in Illinois had chloroform concentrations ranging from less than 1 μg/L to 50 μg/L, with a mean concentration of 10.8 μg/L.

In females, statistically significant increased relative risks of cancer of the large intestine and rectum (OR = 1.19, $P < 0.05$), as well as total digestive tract cancer (excluding liver) (OR = 1.15, $P < 0.05$), were found for chlorinated versus nonchlorinated Illinois groundwater supplies. With respect to total gastrointestinal and urinary tract cancer, the risk was significantly increased in females living within standard metropolitan statistical areas (OR = 1.28, $P < 0.025$) and within urban areas (OR = 1.24, $P < 0.025$) between chlorinated and nonchlorinated groundwater communities.

Where evidence was available concerning a history of chlorination, the authors noted that the relative risk of total gastrointestinal and urinary tract cancer tended to increase with time from initial chlorination, although the change was small. The greatest increase occurred in urban nonstandard metropolitan areas (OR = 1.14 if chlorinated since 1963 and nonsignificant, but OR = 1.28 if chlorinated since 1953 and significant, $P < 0.025$). Although several significant findings were observed in this study, the authors dismissed the results of their own study on the basis that confounding factors such as diet, smoking, and occupation were not controlled. These authors felt that the findings were tenuous and did not confirm the findings of Alavanja et al. (1978) either in strength or in consistency. They state that "chlorination of groundwater does not seem to be a major factor in the etiology of site-specific gastrointestinal and urinary tract cancers."

8.3.7. Struba (1979)

Struba (1979), as part of his Ph.D. thesis, completed a case-control study of mortality in North Carolina on individuals who died at age 45 or under during the period 1975-1978. The cancer sites studied were the rectum, colon, and urinary bladder. Between 700 to 1500 cases per site were matched with controls by age, race, sex, and geoeconomic region (coastal, piedmont, or mountain). Noncancer deaths were excluded if cancer was listed as a contributory or underlying cause of death. For colon and rectal cancer, certain precancerous colonic disorders were excluded (ulcerative colitis, familial polyposis, and adenomatous polyposis). Water data were classified by source, treatment, and previous use. "Source" was defined as ground, surface uncontaminated, or uncontaminated by upstream pollution. "Treatment" was defined as none, prechlorinated, post-chlorinated, or both. "Previous use" included the following 15 categories of upstream pollution for contaminated water only:

(1) Tobacco manufacturing
(2) Textile manufacturing
(3) Textile bleaching and dyeing
(4) Furniture manufacturing
(5) Pulp and paper mills
(6) Chemical industries
(7) Petroleum refining
(8) Rubber and plastics manufacturing
(9) Leather tanning and finishing
(10) Abrassures, asbestos, minerals
(11) Primary metals industries
(12) Electroplating
(13) Electric power generation
(14) Urban areas > 50,000
(15) Out-of-state upstream discharge

The author found small but significant odds ratios (1.3 to 2.0) for all three sites (colon, rectal, and bladder) in rural areas, as well as significant odds ratios for each of the water quality variables in many stratified or combined analyses. Odds ratios for urban areas (population over 10,000) were generally not significant. Urbanization was shown to be an effect modifier for colon cancer and a likely confounder for rectal and bladder cancers. The author considered socioeconomic status to be a likely confounder for cancer of the rectum and bladder. Multivariate analyses showed no evidence that occupation acted as a confounder for bladder cancer in this study. To estimate migration effects, cases and controls were stratified by place of birth and death (birth and death in the same county; birth and death in North Carolina; and death in North Carolina, birth unspecified) and substratified by region, age, race, sex, and urbanization. Odds ratios for treatment (chlorinated and nonchlorinated) were computed for all of the strata. For all three cancer sites, the group with least migratory influence had the highest odds ratio, thus lending support to the author's supposition that an increasing migratory effect is associated with a decreasing risk of cancer of all three sites.

Additionally, Struba found an increasing gradient of risk from the coastal regions of North Carolina to the mountains, a finding that he maintains is consistent with a stronger contrast between surface water and water from deep wells than between surface water and water from shallow wells, which are known to be susceptible to contamination by surface water seepage into groundwater aquifers. However, the author notes that this difference could be due to differences in water treatment practices or confounding by uncontrolled factors such as dietary habits or lifestyles.

8.3.8. Discussion

These later ecological and case-control studies of chlorine exposure and cancer risk from water supplies tend to support the finding of increased risks of bladder, colon, and rectal cancer from exposure to chlorinated water. It seems that this association is at best weak, although significant, as evidenced by odds risk ratios that range as high as 3.6 in the Young et al. (1981) study, but generally fall between 1.1 and 2.0 (see Table 8-16) in the remaining case-control studies. The risk ratios derived in these studies could be explained by the confounding effects of uncontrolled influences such as smoking, diet, air pollution, occupation, and lifestyle, but they appear to have some consistency across several independent and diverse study groups. Of course, all of the case-control studies use residence data and cause-of-death information from death certificates, and thus are not strictly incidence studies. Bias can creep in from several sources: differential survivorship rates due to proximity to better medical care and treatment facilities, higher socioeconomic status, and the possibility of migration of newly diagnosed cancer patients to major medical care centers where chlorination is used to a greater extent. Underestimates of risk can result from failure to control for migration before diagnosis, misclassification of cause of death, and use of chlorination as a surrogate variable in place of more direct measurements of chloroform, especially if the chlorinated source contains few organic contaminants. Hence, the association is weak but significant with regard to the three cancer types and exposure to chlorinated drinking water. Since exposure to chlorine in water is not the same as exposure to chloroform, the most that can be said is that there is a suggestion of an increased risk of cancer of these three sites from exposure to chloroform. If this risk truly exists, it may be due to an

TABLE 8-16. CANCER RISK ODDS RATIOS AND 95% CONFIDENCE INTERVALS (CHLORINATED VERSUS UNCHLORINATED)

Site	Alavanja et al., 1978[a]	Brenniman et al., 1978[b]	Young et al., 1981[c]	Gottlieb et al. 1981[d,e]	Struba 1979[d,e]
Rectum	1.93 (1.31, 2.83)	1.26 (crude) (0.98, 1.61) 1.22 (adjusted)	1.39 high (0.67, 2.86) 1.16 medium (0.58, 2.32) 1.13 low 0.61, 2.08)	1.41 (1.07, 1.87)	1.53 (1.24, 1.89)
Colon	1.61 (1.28, 2.03)	1.08 (crude) (0.96, 1.22) 1.1 (adjusted)	1.51 high (1.06, 2.14) 1.53 medium (1.08, 2.00) 1.53 low (1.11, 2.11)	1.05 (0.95, 1.18)	1.30 (1.13, 1.50)
Bladder	1.69 (1.11, 2.56)	1.04 (crude) (0.81, 1.33) 0.98 (adjusted)	1.04 high (0.43, 2.50) 1.03 medium (0.42, 2.54) 1.06 low (0.60, 3.09)	1.07 (0.84 x 1.36)	1.54 (1.26, 12.88)

[a] Calculated for both sexes and all races combined. Confidence intervals were not stated in Alavanja et al. (1978). Crump (1979) calculated them by applying the method of Fleiss (1979) to data in Alavanja et al. (1978).

[b] Calculated for Caucasians of both sexes. Adjusted values were adjusted for age, sex, urban/rural, and SMSA/nonSMSA. Confidence intervals were not stated in the original report. Crump (1979) calculated them by applying the method of Fleiss (1979) to data on total cases and total controls supplied by Dr. Brenniman in personal communication.

[c] Calculated for white females and for high, medium, and low average daily chlorine doses compared with no chlorination. Odds ratios and confidence intervals computed by logistic regression, controlling for urbanization, marital status, and site-specific occupation.

[d] Calculated for both sexes and all races combined.

[e] Struba (1979) and Gottlieb et al. (1981) also computed odds ratios for surface water versus groundwater as follows. Struba: rectum 1.55 (1.26, 1.91); colon 1.27 (1.10, 1.46); bladder 1.48 (1.22, 1.80). Gottlieb et al.: rectum 1.51 (1.21, 1.90); colon 0.95 (0.88, 1.03).

SOURCE: Crump and Guess, 1982.

intermediate in the natural synthesis of chloroform (communication with Dr. Kenneth P. Cantor, NCI).

In summary, it appears that there may be a suggestion of an increased risk of certain forms of cancer (bladder, large intestine, and especially rectum) due to exposure to chlorinated drinking water contaminated with organic material. The significant associations of cancer at these three sites from chlorinated drinking water (high in organic constituents) does not constitute conclusive evidence of a definite risk of colon/rectal cancer with chloroform, however it is suggestive. The evidence of a significant association of kidney cancer with chloroform exposure in drinking water is even more questionable, since it was based on the findings of only one study, which was confined to males residing in counties where more than 85 percent of the population was served by treated drinking water. A statistically positive correlation was seen only in males residing in counties with over 85 percent treated drinking water. No association was observed in females in these same counties, and the correlations were actually negative for both males and females in counties with less than 85 percent treated drinking water.

It appears that these case-control and ecological studies in humans suggest a weak but significant association of certain forms of cancer (colon, bladder, and especially rectal) with chlorinated drinking water contaminated with organic material. Chloroform appears to be the single largest constituent, but further epidemiologic research should be accomplished to confirm these findings.

In view of what appears to be common problems with the many case-control and ecological studies of chloroform, it seems appropriate to employ a study design such that these common problems could be eliminated or at least reduced in scope. Such a suggested design is the retrospective prospective cohort design. Ideally, the design calls for the identification of a large group (say 1000) of individuals who were exposed to only chloroform sometime in the past. The constructed cohort would then be followed through time from date of initial employment in the job in which the exposure occurred to the present and adverse health consequences occurring to the cohort during this follow-up period would be compared with that of the general population.

Since hospital, laboratory, and university personnel used chloroform in the past as an anesthetic, the feasibility of constructing a cohort from personnel records of exposed individuals (i.e., lab technicians, anesthesiologists, and nurses) maintained by these institutions should be examined if they can be shown to have been exposed to chloroform. One problem, however, that might remain is the possibility of the occurrence of concomitant exposures to other substances that may be carcinogenic, though presumably the profile of potential exposures would be different from that found in all the drinking water studies. This possibility can be evaluated at the time. The presumed route of exposure would be absorption and/or inhalation in humans rather than ingestion, as it was in all the earlier drinking water studies.

8.4. RISK ESTIMATES FROM ANIMAL DATA

Evidence for the carcinogenicity of chloroform consists of several positive long-term studies in mice and rats (NCI, 1976; Roe et al., 1979; Jorgenson et al., 1985). These positive animal studies report a dose-dependent excess incidence of liver carcinomas in male and female mice, and of malignant renal tumors in mice and rats. There is also indication (from binding studies and from mutagenicity tests that utilize endogenous or _in vivo_ metabolism) that chloroform may have the potential to be a weak mutagen. Metabolism produces phosgene and other putative reactive metabolites that covalently bind extensively to cellular lipids and proteins. Binding _in vivo_ to DNA, although at low levels, has been demonstrated. Organ localization and binding intensity parallel acute cellular toxicity in liver and kidney of experimental animals. There are no known qualitative differences among species with regard to metabolic pathways or metabolite profiles.

It is important to note that the quantitative estimations of the impact of chloroform as a carcinogen are made independently of the overall weight of evidence that chloroform is carcinogenic in animals. The calculations are made as if chloroform were a human carcinogen.

8.4.1 Possible Mechanisms Leading to a Carcinogenic Response for Chloroform

Possible mechanisms proposed for carcinogenesis include direct interaction of a chemical or its metabolites with DNA, long-term tissue injury, stimulation of cell proliferation, immunosuppression, hormonal imbalances, or release of altered cells from growth control (Weisburger and Williams, 1980, 1981). The current knowledge of chloroform metabolism and the acute cellular toxicity of its reactive intermediate metabolites suggest several different general cellular processes that possibly may lead to carcinogenic activity (Davidson et al., 1982). One process may involve cell death induced by the cellular toxicity of chloroform followed by the consequent stimulation of DNA replication associated with cell multiplication resulting in an indirect or promoting effect. The continuing process of DNA replication results in increased proportions of single strand DNA, which is more susceptible to irreversible binding by reactive intermediates than is the double strand DNA. Or, it is possible that chloroform acts by stressing the fidelity of replication, increasing the possibility of introducing DNA transcript errors. This general process is supported by the carcinogenicity studies of Eschenbrenner and Miller (1945) and also Roe et al. (1979), as well as by the sites of observed tumors. The location of chloroform-induced primary tumors closely follows the sex and species specificity seen in the acute organ toxicity as described by many investigators (see section on toxicity). Eschenbrenner and Miller proposed that necrosis constituted a necessary precursor for chloroform liver carcinogenicity. This process is consistent with a threshold effect at the necrotizing doses at which increased cell death and turnover occur. It is also consistent with the lack of covalent binding of chloroform or its metabolites to nucleic acid as observed by Uehleke and coworkers (1977) and by Diaz and Castro (1980), as well as the negative results of the majority of bacterial mutagenicity assays (see Chapter 7 on mutagenicity).

A second possible process may involve a suppression of certain homeostatic mechanisms which maintain cellular integrity. For example, depletion of a cellular detoxification compound like glutathione could be expected to raise the background tumor incidence. If this type of process were the only mechanism involved in chloroform carcinogenicity, a nonlinear relationship between tumor incidence and exposure dose would be expected, probably with a threshold dose below which no tumors would occur. This possible carcinogenic process is supported by the work of Elkstrom and Hogberg (1980) and others (Brown et al., 1974; Docks and Krishna, 1976), who have observed that glutathione depletion occurs in chloroform-treated hepatocytes *in vitro* as well as *in vivo*. Such depletion may reduce the availability of glutathione for detoxification of certain reactive intermediates of chloroform metabolism as well as other chemical species.

Another general process that may lead to carcinogenicity involves the metabolic production of active chloroform intermediate metabolites (phosgene, carbenes, and free radicals) and their direct interaction with DNA. This genotoxic process could be expected to be modulated by various homeostatic mechanisms, such as DNA repair and immunological surveillance, but it is generally regarded as lacking a threshold. This mechanism is supported by the data of Agustin and Lim-Sylianco (1978), who found that chloroform produced a positive result in the micronucleus test and host-mediated mutagenicity assays in the mouse. Also, the positive mutagenicity results of Callen et al. (1980) in yeast, the observations of abnormal sperm morphology in chloroform-treated mice (Land et al., 1981), and sister chromatid exchange in human lymphocytes and mouse marrow (Morimoto and Koizumi, 1983) provide support for a genotoxic mechanism. Furthermore, the data available from certain epidemiology studies (Linde and Mesnick, 1980; Cantor et al., 1978; Hogan et al., 1979) can be considered to be in accord with this process.

In the absence of definitive evidence solely supporting any one of the likely processes operative in the carcinogenic activity of chloroform, the risk assessment performed by the Carcinogen Assessment Group considers the process which is generally accepted to be associated with the greatest risk--the genotoxic mechanism. The risk of chloroform carcinogenicity by this process, with its lack of threshold, is appropriately estimated by a mathematical model predicting zero incremental risk only at zero exposure. In addition, at the present time, the lack of sufficient understanding about mechanism of action involved in carcinogenesis does not allow for a distinction between chemicals acting directly with DNA and those which do not, nor can those which have not been shown to be genotoxic be considered to have identifiable population thresholds or be safer than those which are considered to be genotoxic (Pereira, 1984). More research is required on the mechanism of action of chloroform before a threshold model would be appropriate for carcinogen risk assessment (IARC, 1983; Pereira, 1984).

8.4.2. Selection of Animal Data Sets

For chloroform, several studies in different animal species, strains, and sexes, run at several doses and different routes of exposure, are available. A choice must be made as to which data set(s) from these several studies to use in the risk model. It may also be appropriate to correct for differences in metabolism between species, and for different routes of administration. The procedures used herein in evaluating such data are consistent with the approach of making a maximum-likelihood risk estimate.

The following studies, selected as evidence of the carcinogenic activity of chloroform from lifetime treatment studies in laboratory animals, have been used in the mathematical extrapolation models.

8.4.2.1. NCI 1976 Bioassay (Mice): Liver Tumors (Table 8-17)--Male and female mice were divided into two treatment groups of 50 animals per sex per dose. A matched control group of 20 animals per sex, and pooled control groups of 77 males and 80 females, were included in the study. Initially, male mice received doses of 100 and 200 mg/kg in corn oil by gavage, while females received 200 and 400 mg/kg beginning at 5 weeks of age, 5 days/week for 78 weeks, with sacrifice at 92-93 weeks. However, after 18 weeks, these doses were increased to 150 and 200 mg/kg for males and 250 and 500 mg/kg for females. The time-weighted average doses were 138 and 277 mg/kg for males and 237 and 477 mg/kg for females. Mean body weights at terminus were approximately 35 g for males and 28 g for females. (See Section 8.1 for more complete details.)

TABLE 8-17. INCIDENCE OF TUMORS IN EXPERIMENTAL ANIMAL STUDIES

Subjects	Dose, mg/kg/day	Tumor Type	Incidence rate(%)
B6C3F1 mice (female)[a]	0	Hepatocellular carcinoma	0/20 (0%)
	238		36/45 (80%)
	477		39/41 (95%)
B6C3F1 mice (male)[a]	0	Hepatocellular carcinoma	1/18 (6%)
	138		18/50 (36%)
	277		44/45 (98%)
Osborne-Mendel rats, (male)[b]	0	Renal tubular cell adenocarcinoma	0/19 (0%)
	90		2/50 (4%)
	180		10/50 (20%)
ICI mice, (male)[c]	0	Malignant kidney tumors	0/50 (0%)
	60		9/48 (19%)
Osborne-Mendel rats, (male)[d]	0	Renal tubular cell adenomas and carcinomas	4/301 (1%)
	19		4/313 (1%)
	38		4/148 (3%)
	81		3/48 (6%)
	160		7/50 (14%)

[a] NCI, 1976.
[b] NCI, 1976.
[c] Roe et al., 1979
[d] Jorgenson et al., 1985

8.4.2.2. NCI 1976 Bioassay (Rats): Kidney Tumors (Table 8-17)--Chloroform solutions in corn oil were given by gavage at dose levels of 90 and 180 mg/kg. Fifty male rats were treated at each dose, with colony control groups (99) consisting of four vehicle-control groups, including matched controls, given corn oil. The chronic study started at 52 days (7 weeks) of age and ended with sacrifice of survivors at 111 weeks. Chloroform was administered in corn oil 5 days/week for the initial 78 weeks. Initial body weights were about 250 g; by 100 weeks, mean body weights were approximately

500 g in controls and treated groups. (See pages 8-2 to 8-8 for fuller details.)

8.4.2.3. Roe et al. 1979 Bioassay (Mice): Kidney Tumors (Table 8-17)--These investigators conducted three experiments on the carcinogenicity of chloroform in mice of four strains. Males of the ICI strain were the only animals that showed an increased incidence of tumors. In the first two experiments, chloroform in toothpaste vehicle was given by gavage. In the third experiment, chloroform in arachis oil was tested in ICI mice by gavage at a dose level of 60 mg/kg/day. Treated and control groups were each composed of 52 male ICI mice. Chloroform was given by gavage 6 days/week for 80 weeks, beginning at 10 weeks of age and followed by an observation period of 13 to 24 weeks. Controls were given arachis oil alone. (See Section 8.1.2.2 for fuller details.)

8.4.2.4. Jorgenson et al., 1985 Bioassay (Rats): Kidney Tumors (Table 8-17) --Chloroform was administered in the drinking water of male rats and female mice at concentrations of 200, 400, 900, and 1800 mg/L. Only the rats showed an increased incidence of tumors. The rats were started on treatment at an average age of 7 weeks and continued on study for 104 weeks. The doses supplied to the rats in their drinking water correspond to time-weighted average doses of 19, 38, 81, and 160 mg/kg body weight. The dose-related increase of renal tumors in the male rats is consistent with the findings of the earlier NCI (1976) study. However, the lack of response in the mice when chloroform was administered in the drinking water suggests that earlier reports of chloroform hepatocellular carcinoma in mice may be related in some way to the dosing regimen, absorption patterns, or peak blood and tissue levels of chloroform and its reactive metabolites. Chloroform administered in corn oil provides lower peak blood levels than the same dose of chloroform administered in aqueous solution (see Chapter 4). However, the daily single bolus dose given by gavage would be expected to have different pharmacokinetic characteristics than a similar amount of chloroform administered in smaller intermittent doses over the twenty-four hour day. The corn oil carrier used in the NCI gavage study has not been shown to induce an increase in the incidence of liver tumors in mice, although this has been postulated.

The studies discussed (see Table 8-17) indicate that the carcinogenic response to chloroform may be strain-, species-, and sex-related, in addition to being organ-specific primarily for liver and kidney, which are the target organs of acute chloroform toxicity, metabolism, and covalent binding. All long-term studies, with chloroform administered as a single daily oral dose, have shown a dose-related increased incidence of neoplasms in two strains of mice, both sexes of one strain and female mice of another strain, renal neoplasms in male mice of a third strain, and hepatomas in mice of a fourth strain (sex not reported). When tested in two rat strains, chloroform produced renal neoplasms in male animals of one strain only (Osborne-Mendel). Roe et al. (1979) noted that the acute oral LD_{50} in the B6C3F1 strain of the NCI study was approximately double that of the four strains of their study, and hence they suggested that the increased incidence of liver tumors in both male and female mice of the NCI B6C3F1 strain may be related to the higher doses used in the NCI mouse study (Table 8-17). Chloroform nephrotoxicity was also found to be greater in the male ICI mouse strain, suggesting a greater susceptibility of this sex and strain to cellular damage possibly associated with a carcinogenic response. However, in the Eschenbrenner and Miller (1945) strain A mouse study, only the males developed renal necrosis but without renal tumor formation upon repeated oral administration of chloroform.

8.4.3. Interspecies Dose Conversion

8.4.3.1. General Considerations--Carcinogenesis is a complex process, not entirely understood, and no observational evidence exists to validate satisfactorily the extapolated predictions of carcinogenesis from animal models to humans. Thus, there is no scientific basis per se for choosing one extrapolation method over another in extrapolation of the carcinogenic response. However, in extrapolating the dose-carcinogenic response relationships of laboratory animals to humans, the doses used in the bioassays must be adjusted in some way to allow for such differences as size and metabolic rates. Therefore, other biological information, particularly interspecies data, is examined for chloroform, since physiologic, biochemical, and toxicologic responses other than cancer may provide information as to what factors might be considered and what method is generally appropriate for the extrapolation from laboratory animals to humans for this chemical. The major components requiring consideration in determining an appropriate extrapolation base for scaling carcinogenicity data in laboratory animals to humans are 1) toxicological data, 2) covalent binding, and 3) metabolism and kinetics. The biological basis for extrapolation of dose-carcinogenic response relationships has been outlined and discussed previously (Davidson, 1984; Parker and Davidson, 1984).

8.4.3.1.1. Toxicologic data. There is a wealth of toxicologic data derived from numerous studies on the mechanisms of chloroform toxicity (Chapter 5). Chloroform, as an anesthetic, has a depressive effect on the central nervous system (CNS) leading to respiratory depression and death. As CNS depression tends to occur at similar blood and tissue concentrations across species, the acute LD_{50} tends to be similar. Other toxicologic endpoints have been measured, but these measures (for example, lowest effective dose producing liver and kidney pathology) have been made in numerous separate studies and are difficult to evaluate comparatively across animal species.

It has been well established, however, that chloroform produces liver and kidney toxicity across species, i.e., man, rat, and mouse. The striking experimental observation is that even within a species, strain and sex differences determine the "susceptibility" for liver and kidney toxicity to a fixed dose of chloroform (Eschenbrenner and Miller, 1945; Culliford and Hewitt, 1957; Hill et al., 1975). These differences reflect differences of magnitude of chloroform metabolism as well as other factors at these target organs (see Chapter 4), and the extent of covalent binding parallels the LD_{50} and renal and liver cytotoxicity observed with strain and sex (Hill et al., 1975).

The species and sex differences observed for the carcinogenic response to chloroform (Table 8-17) are difficult to explain solely on the basis of genotoxicity, unless it is assumed metabolism differs with species and sex. On the other hand, the acceptance of a non-genetic mechanism of tumor induction (from repeated cellular damage resulting in enhanced cellular regeneration with increased frequency of gene miscoding or promotion of background tumor cells, etc.) might suggest that the carcinogenic response to chloroform in laboratory animals, dependent as it is on the dose, species, strain, and sex (Table 8-17), cannot be readily extrapolated to man (apart from problems of "threshold"). However, the human population encompasses a full range of genetic variability, in contrast to the inbred strains of laboratory animals, and, hence, a carcinogenic response observed only in certain species, strains, or sexes in laboratory animals may be expected to be expressed in the human counterpart.

8.4.3.1.2. Covalent binding--The metabolism of chloroform in mammals gives rise to reactive intermediate metabolites, including phosgene and, possibly, carbene and chloride radicals. The irreversible binding to cellular macromolecules of these reactive chemical species is generally believed to be responsible not only for hepatic and renal damage from chloroform, but also for the carcinogenic response, although the mechanism for the latter has not been elucidated. The data of Figure 8-4 from Ilett et al. (1973) show that in C57BL mice the amount of covalent binding increases with dose up to about 450 mg/kg, which seems to be a "saturation of metabolism" dose for these mice. Furthermore, the amount of covalent binding from metabolism parallels the dose-response curve for cytopathologic changes associated with acute toxicity. Uehleke and Werner (1975) (Figure 8-5) found that, across species (mouse, rat, rabbit, man), irreversible binding from chloroform metabolism, as measured with hepatic microsomes from these species, was greater in humans than in rabbits, and was greater in rabbits than in rodents. This indicates that the capacity to metabolize chloroform/unit of microsomes is much greater in man than rodent. That is, while liver weights between species are proportional to body weight ($W^{1.0}$), the greater activity of microsome/unit weight in the metabolism of chloroform indicates that man is certainly not "limited" in metabolic capacity as compared with rodents, and indeed, may have a capacity greater than expected from the usual extrapolations on body weight or surface area.

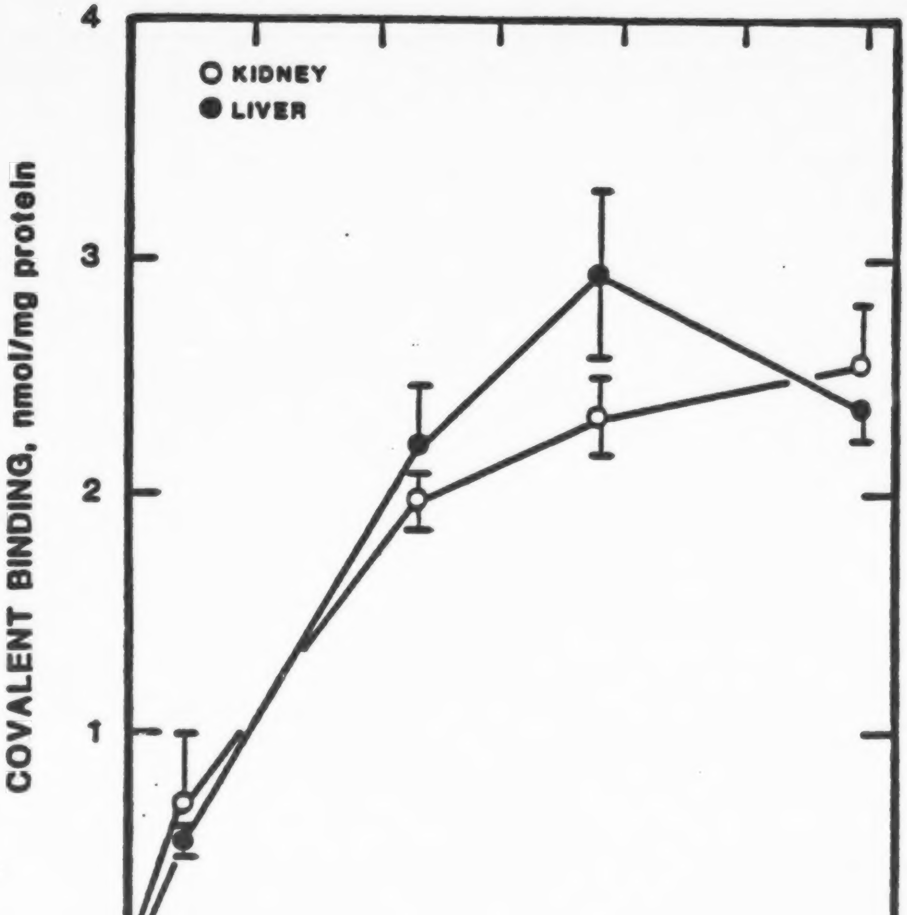

Figure 8-4. Effect of increasing dosage of i.p.-injected ^{14}C-chloroform on extent of covalent binding of radioactivity in vivo to liver and kidney proteins of male mice 6 hours after administration.

Source: Ilett et al. (1973).

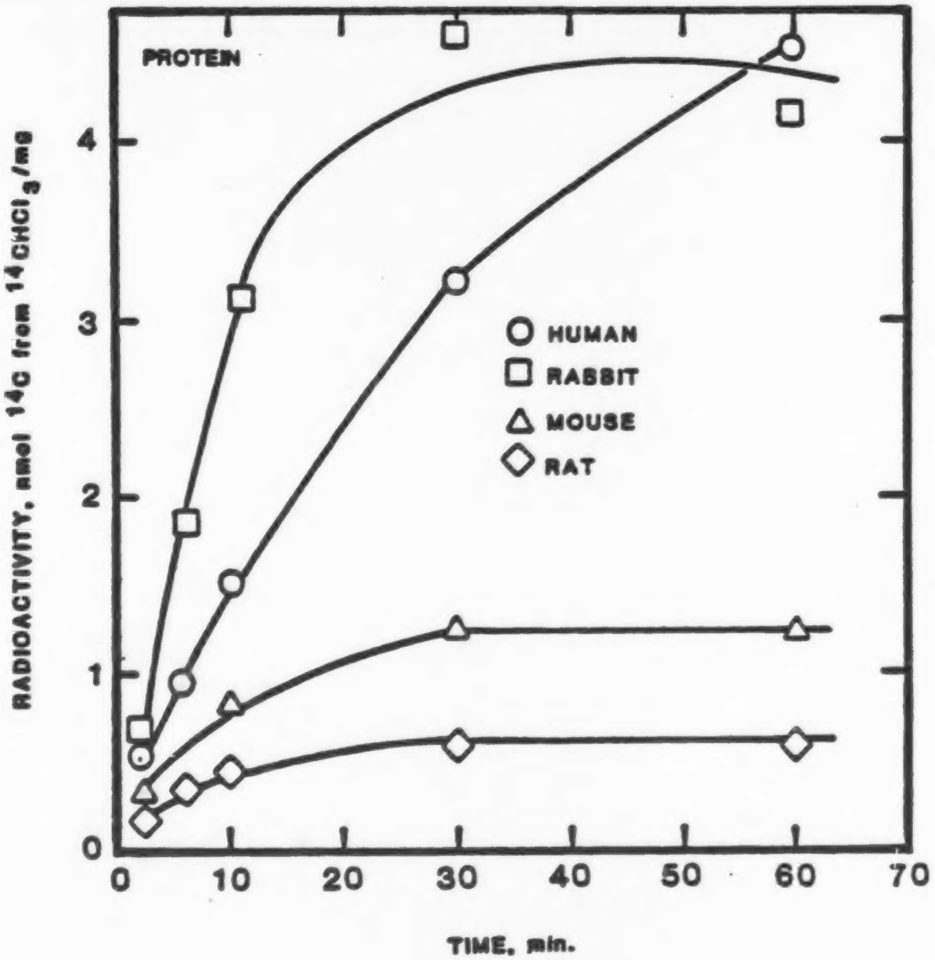

Figure 8-5. Comparison of irreversible binding of radioactivity from ^{14}C-$CHCl_3$ to protein and lipid of microsomes from normal rabbit, rat, mouse, and human liver incubated *in vitro* at 37°C in O_2.

Source: Uehleke and Werner (1975).

A basic question concerning chloroform is whether it has genotoxicity potential, i.e., whether covalent binding to cellular DNA occurs. Most investigators have found only minimal covalent binding to DNA (p. 4-58). Reitz et al. (1980) measured DNA covalent binding in liver and kidneys of mice after an oral dose of 240 mg/kg of ^{14}C-chloroform. These investigators found limited evidence that the metabolism of chloroform by the mouse in vivo results in genotoxicity, i.e., detectable DNA alkylation. DNA alkylation represented a binding index, CBI, of 1.5, a value well above the detection limit after correction for background. The remaining question of whether the degree of chloroform alkylation represents a highly significant genotoxicity as opposed to "very low" DNA aklylation (as judged by Reitz et al.), depends in large part on the validity of the experiments' methodology. Reitz et al. include a direct comparison, in tabular form, of the alkylation of chloroform with that of potent alkylating carcinogens. However, their chloroform DNA alkylation experiment was not actually conducted with these "positive controls." An overall conclusion can be made that while DNA alkylation did occur, the comparative extent is open to question.

8.4.3.1.3. Metabolism and kinetics

8.4.3.1.3.1. Absorption--Chloroform is virtually completely absorbed from the gastrointestinal tract when given by gavage in olive oil (60 mg/kg) to mice, rats, and monkeys, and when given orally to man (0.1 to 1.0 g) (Brown et al., 1974; Taylor et al., 1974; Fry et al., 1972). Withey et al. (1982) observed that chloroform given in a corn oil vehicle was absorbed more slowly than chloroform given in water. Nonetheless, the experimental data support complete absorption of chloroform dosage for the conditions of the NCI, 1976; Roe et al., 1979; and Jorgenson et al., 1985 carcinogenicity studies and show no difference in absorption characteristics among species, including man.

8.4.3.1.3.2. First-pass effect--Chloroform, after gavage to animals or oral ingestion by humans, is absorbed and transported by the portal blood flow to the liver, where a portion is extracted and metabolized (depending on dose) and a portion excreted through the lungs. In both animals and humans, pulmonary excretion of unchanged chloroform is dose-dependent (Tables 8-18 and 8-19). Data are available (Table 8-18) from the studies of Brown et al. (1974) for the pulmonary excretion in mice and rats after an oral dose of 60 mg/kg (6 percent and 20 percent of the dose, respectively). Pulmonary excretion is the principal route of excretion of unmetabolized chloroform. Therefore, the amounts of chloroform metabolized possibly contributing to the carcinogenic response after a 60-mg/kg dose are 56.4 mg/kg for mice and 48.9 mg/kg for rats. For higher doses of the NCI studies, experimental data are not available, and hence the body burdens must be calculated using 94 percent and 80 percent for the gavage dose to mice and rats, respectively, although the resultant calculated body burdens will be higher than in actuality because the portion (percent) of the dose excreted unchanged through the lungs increases with the oral dose (see data for humans, Table 8-19).

8.4.3.1.3.3. Saturation of metabolism--The mammalian capacity to metabolize a chemical compound may nearly always be "saturated" if the dose to the organism is high enough. Chloroform is no exception. While there are not specific experimental data to illustrate chloroform saturation kinetics in laboratory animals, the amount of covalent binding versus ^{14}C-chloroform dose from the data of Ilett et al. (1979) (Fig. 8-4) suggests in mice a saturation of metabolism at about 75 mg/kg/hour. Fry et al. (1972) conducted

TABLE 8-18. SPECIES DIFFERENCE IN THE METABOLISM OF ^{14}C-CHLOROFORM (ORAL DOSE OF 60 mg/kg)[a]

Species	No.	^{14}C-radioactivity 48 hours after dose Mean values as percent dose				
		Expired $^{14}CHCl_3$ or metabolites	Expired $^{14}CO_2$	Urine + feces	Carcass	Total
Mice CF/LP, CBA, C57 strains	19	6.1	85.1	2.6	1.8	95.6
Rats S-D	6	19.7	65.9	7.6	NR	93.2
Squirrel Monkeys	6	78.7	17.6	2.0	NR	98.3

[a] Recalculated from the data of Brown et al., 1974.
NR = Not recorded

TABLE 8-19. PULMONARY EXCRETION OF CHLOROFORM FOLLOWING ORAL DOSE

Excretion of $^{13}CHCl_3$: Percent of Dose[a]

Subjects	Dose[a] (g)	Mean for 8 hours[b]	Range
8 M and F	0.5	40.3	17.8 to 66.6
1	1.0	64.7	NA
1	0.25	12.4	NA
1	0.10	nil	NA

Excretion of $^{13}CO_2$ following 0.5-g dose of $^{13}CHCl_3$: Cumulative percent of dose[b]

Subjects	Time after dose				
	0.5	1.75	2.5	5.5	7.6
Male (1)	2.1	24.1	35.9	49.2	50.6
Female (62.7 kg) (1)	0.5	10.7	28.3	47.5	48.5

[a] Recalculated from the data of Fry et al., 1972.
[b] Within 4% of value calculated for infinite time.
NA = Not applicable.

studies in man (Table 8-19) that indicate saturation of metabolism in man at relatively low doses. These investigtors administered chloroform orally to volunteers over a dose range of 0.10 to 1.0 g (about 1.5 to 15 mg/kg), and found that the portion of the dose excreted unchanged via the lungs increased from zero for the lowest dose to 65% with the highest dose. These results suggest that chloroform metabolism is rate-limited in man. Since a diminishing proportion of dose is metabolized with increased dose, the V_{max} for man appears to approximate 50 mg/hour or 0.7 mg/kg/hour as estimated from the highest dose, 15 mg/kg.

8.4.3.1.4. Interspecies scaling of metabolism--From the data of Brown et al. (1974) and of Fry et al. (1972), which provide information on the metabolism of chloroform in mice (3 strains), rats, squirrel monkeys, and man, it is possible to estimate the amount metabolized (mg) of an orally administered common dose of 60 mg/kg body weight (probably a near saturating dose; see above). These data, when plotted as the logarithm of the amount metabolized versus the logarithm of the species body weight in accordance with the allometric relationship $Y = aW^n$ ($\ell n\ Y = \ell n\ a + n\ell n\ W$), gives a regression line that closely fits the species data points (Fig. 8-6). The slope of the line, n = 0.65, provides strong evidence that chloroform metabolism in these four species is proportional to their surface areas ($W^{2/3}$). Figure 8-6 provides justification for the extrapolation of the carcinogenic response (metabolism-dependent) of mice and rats of the Jorgenson, Roe, and NCI studies to man on the basis of body surface area ($W^{2/3}$).

8.4.3.2. Calculation of Human Equivalent Doses--Available information on metabolism and pharmacokinetics pertinent to the conditions of the Jorgenson et al., 1985; Roe et al., 1979; and NCI, 1976 carcinogenicity assays may be summarized as follows:

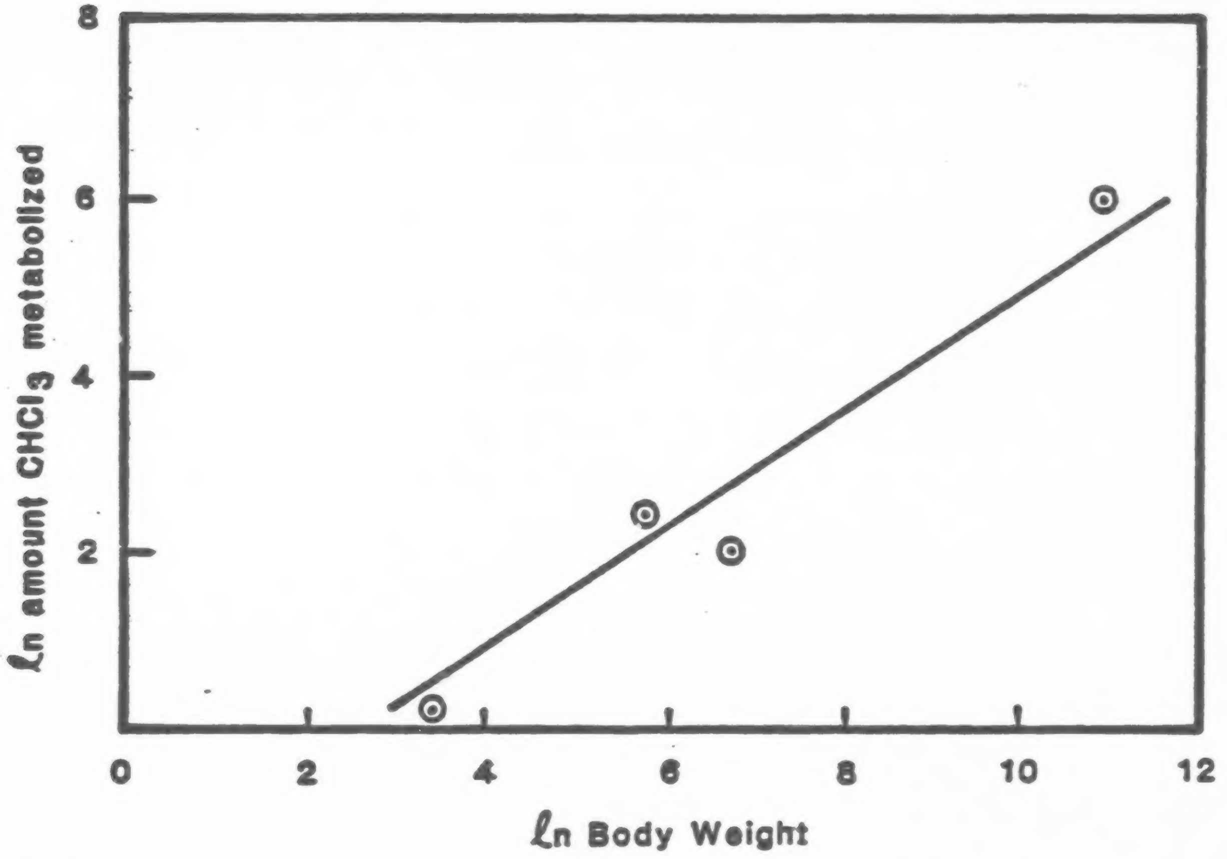

Figure 8-6. Allometric relationship ($Y = aW^n$) between species body weight (in order: mouse, rat, squirrel monkey, and man) and the amount metabolized of a common oral dose of chloroform as calculated from the data of Fry et al. (1972) and Brown et al. (1974). The species body weights assumed were: mouse, 30 g; rat, 300 g; squirrel monkey, 850 g; and man, 70 kg. The slope of the regression line is 0.65, indicating that metabolism of chloroform in these species is proportional to their surface area.

1. After gavage or other oral administration to mice and rats or oral administration to man, chloroform is rapidly and virtually completely absorbed.

2. After gavage or oral administration, a portion of the dose (increasing with the dose) is excreted via the lungs unchanged. Metabolism of chloroform is "saturated" or near saturation at the dose levels of the bioassays in which the chloroform was administered as a single daily bolus. The effective dose (amount metabolized to reactive metabolites) is the gavage dose minus the percentage of dose excreted unchanged. For mice given 60 mg/kg (conditions of Roe study), this is about 6 percent, and for rats given the same dose, 20 percent. However, for the NCI study the doses approximate 200 to 500 mg/kg/day for rats and mice, and hence a 20 percent correction is very conservative and probably leads to an overestimate of the amounts metabolized from these doses. Virtually all of the chloroform administered in the drinking water study is metabolized due to the dosing regimen.

3. The adipose tissue/blood partition coefficient for chloroform is high (35), and the half-time of chloroform residence in adipose tissue is relatively long in the rat and man (about 2 hr and 36 hr, respectively); daily gavage doses may not be expected to be completely cleared from the body within the 24-hr dosing interval, and therefore chronic daily dosing, as occurs in the animal bioassays, may result in the constant presence of chloroform (and metabolites) in the body (except for weekends).

4. There is an experimental basis for determining that the metabolism of chloroform across species (including man) is proportional to the surface area of the species ($W^{2/3}$).

5. There is no evidence to suggest any qualitative difference in the metabolic pathways of the three species (mouse, rat, and man) for chloroform.

6. In mice, covalent binding (from chloroform metabolism) has been demonstrated in both liver and kidney (target organs) to be proportional to the dose.

The total information available on the pharmacokinetics and metabolism of chloroform in the mouse and rat is sparse and does not include information for doses that bracket the high doses of the NCI carcinogenicity assay, although such information is available for the doses used in the Roe et al. assay. Nonetheless, the information on kinetics and metabolism justifies the use of surface area ($W^{2/3}$) as a basis for dose extrapolation from experimental animals to man and the calculation of human equivalent doses (see Figure 8-6).

Using this pharmacokinetic and metabolic information, the lifetime average human equivalent doses for the chloroform NCI mouse and rat carcinogenicity bioassays (1976), for the chloroform-Roe et al. ICI mouse carcinogenicity bioassay (1979), and for the chloroform in drinking water-Jorgenson et al. rat carcinogenicity bioassay (1985), are calculated as follows. The lifetime average daily exposure (LAE) of bioassay animals is given by

$$\mathrm{LAE_A} = \frac{\text{duration exposure (wk)}}{\text{duration experiment (wk)}} \times \frac{\text{doses per wk}}{7} \times \text{effective dose, mg/animal/day}$$

where the effective dose is the average assay dose per day in milligrams, adjusted for pharmacokinetic and metabolic parameters (in this case decreased by 6 percent for mice and by 20 percent for assay rats administered single bolus daily doses). For humans, the LAE is given as follows:

$$\text{LAE}_H = \text{LAE}_M \times \text{scaling factor.}$$

For chloroform, the appropriate basis for extrapolation from mouse or rat to man has been determined from the experimental data to be that of body surface area ($W^{2/3}$). Therefore, the scaling factor is

$$\left(\frac{\text{body weight of man (70 kg)}}{\text{terminal body weight of assay animal (kg)}}\right)^{\frac{2}{3}}$$

Tables 8-20 through 8-23 show, respectively, the calculated lifetime average human equivalent doses (LAE_H) with corresponding tumor incidence for the chloroform-NCI mouse bioassay, the chloroform-NCI rat bioassay, the chloroform-Roe et al. mouse bioassay, and the chloroform-Jorgenson et al. rat bioassay.

Figure 8-7 shows visually the relationship between the equivalent human exposure dose (LAE_H) and bioassay tumor incidence. Dose expression in the form of human equivalent dose effectively "normalizes" the varying conditions of the individual bioassays, particularly species differences, to the single human standard, hence facilitating a common dose-response comparison, as shown in Figure 8-7. It should also be noted that, whereas in Figure 8-7 the equivalent human dose in units of mg/m^2/day is plotted, plots of equivalent human dose in units of mg/day or (mg/kg)/day provide dose-response relationships of an entirely similar form since all three dose units (mg/day, mg/kg/day, and (mg/m^2)/day) are directly proportional and interconvertible

TABLE 8-20. CONTINUOUS HUMAN EQUIVALENT DOSES AND INCIDENCE OF HEPATOCELLULAR CARCINOMAS IN MALE AND FEMALE B6C3F1 MICE

Gavage dose, mouse (mg/kg)[b]	Lifetime average daily dose (mg/mouse)	Equivalent lifetime average human exposure dose[a]			Tumor incidence (%)
		(mg/d)	(mg/m^2)/d	(mg/kg)/d	
0, female	--	--	--	--	0/20 (0)
238, female	3.74	694.7	375.5	9.9	36/45 (80)
477, female	7.61	1394.5	753.8	19.9	39/41 (95)
0, male	--	--	--	--	1/18 (6)
138, male	2.75	436.2	235.8	6.2	18/50 (36)
277, male	5.52	876.3	473.7	12.5	44/45 (98)

[a] Where the terminal average weight of the male mice is 0.035 kg, female mice is 0.028 kg,and standard man, 70 kg with 1.85 m^2 surface area. Mouse-to-man extrapolation factor is $(70/0.035)^{2/3}$ for male mice and $(70/0.028)^{2/3}$ for female mice.

[b] Sum of all doses divided by number of days dosed.

[c] Lifetime average daily dose = mg/kg dose x body weight x 5/7 days x 78/91 weeks (corrected for 6% dose unmetabolized).

SOURCE: NCI, 1976.

TABLE 8-21. CONTINUOUS HUMAN EQUIVALENT DOSES AND INCIDENCE OF RENAL TUBULAR-CELL ADENOCARCINOMAS IN MALE OSBORNE-MENDEL RATS

Gavage dose, rat (mg/kg)	Lifetime average daily dose (mg/rat)	Equivalent lifetime average human exposure dose[a]			Tumor incidence (%)
		(mg/d)	(mg/m^2)/d	(mg/kg)/d	
0, male	--	--	--	--	0/99 (0)
90, male	18.07	487.0	263.2	6.96	4/50 (4)
180, male	36.14	973.9	526.4	13.92	12/50 (20)

[a] Where the terminal average weight of the male rats is 0.5 kg and standard man, 70 kg with 1.85 m^2 surface area. Rat-to-man extrapolation factor is $(70/0.5)^{2/3}$.

[b] Lifetime average daily dose = mg/kg dose x body weight x 5/7 days x 78/111 weeks (corrected for 20% dose unmetabolized).

SOURCE: NCI, 1976.

TABLE 8-22. CONTINUOUS HUMAN EQUIVALENT DOSES AND INCIDENCE OF MALIGNANT KIDNEY TUMORS IN MALE ICI MICE

Gavage dose, mouse (mg/kg)	Lifetime average daily dose[b] (mg/mouse)	Equivalent lifetime average human exposure dose[a]			Tumor incidence %
		(mg/d)	(mg/m^2)/d	(mg/kg)/d	
0, male	--	--	--	--	0/50 (0)
60, male	1.45	229.4	124.0	3.38	9/48 (19)

[a] Where the terminal average weight of the male mouse is assumed to be 0.035 kg and standard man, 70 kg with 1.85 m^2 surface area. Mouse-to-man extrapolation factor is $(70/0.035)^{2/3}$.

[b] Lifetime average daily dose = mg/kg dose x body weight x 6/7 days x 80/93 weeks (corrected for 6% dose unmetabolized).

SOURCE: Roe et al., 1979.

TABLE 8-23. CONTINUOUS HUMAN EQUIVALENT DOSES AND INCIDENCE OF RENAL TUBULAR CELL ADENOMAS AND ADENOCARCINOMAS IN MALE OSBORNE-MENDEL RATS

Dose[a]	Lifetime average daily dose (mg/rat)	Equivalent lifetime average human exposure dose[a]			Tumor incidence %
		(mg/d)	(mg/m^2)/d	(mg/kg)/d	
0	--	--	--	--	4/301 (1)
19	8.9	240	130	3.43	4/313 (1)
38	19.8	480	257	6.86	4/148 (3)
81	42.2	1038	561	14.83	3/48 (6)
160	84.4	2021	1094	28.87	7/50 (14)

[a] Where the terminal average weight of the male rat is 0.5 kg and the standard man 70 kg with 1.85 m^2 surface area. Mouse-to-man extrapolation factor is $(70/0.035)^{2/3}$.

[b] Lifetime average daily dose = mg/kg dose x body weight x 7/7 days x 104/111 weeks (corrected for 0% dose unmetabolized).

SOURCE: Jorgenson et al., 1979.

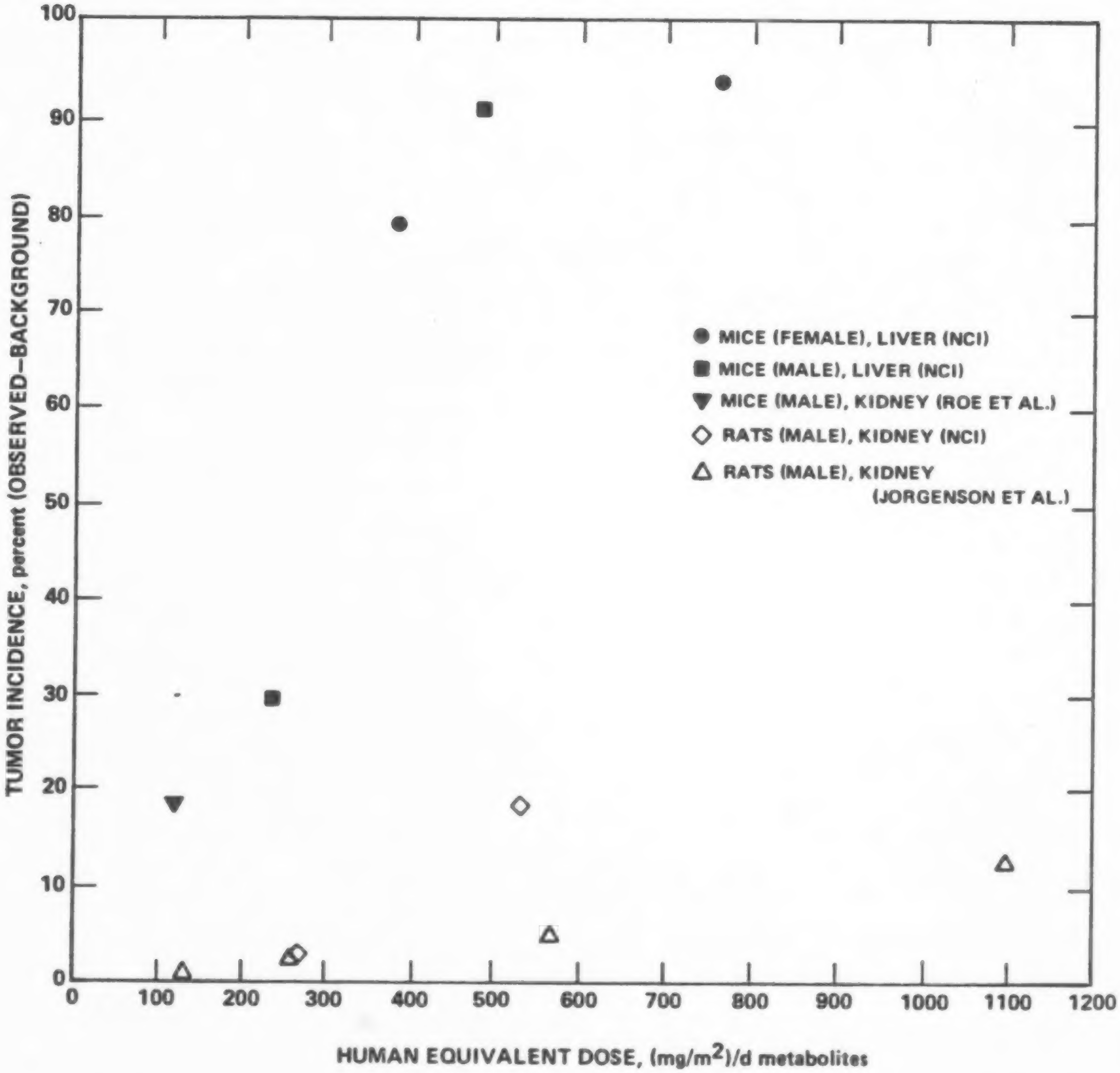

Figure 8-7. The relationship between the equivalent human exposure dose and bioassay tumor incidence.

one to another by means of the conversion factors of 70 kg and 1.85 m^2 surface area for the "standard" man.

8.4.4. Choice of Risk Model

8.4.4.1. General Considerations--The data used by the CAG for quantitative estimation are of two types: 1) lifetime animal studies and 2) human studies where excess cancer risk has been associated with exposure to the agent. In animal studies it is assumed, unless evidence exists to the contrary, that if a carcinogenic response occurs at the dose levels used in the study, then responses will also occur at all lower doses, with an incidence determined by the extrapolation model.

There is no universally acceptable solid scientific basis for any mathematical extrapolation model that relates exposure to cancer risk at the extremely low concentrations that must be dealt with in evaluating environmental hazards. For practical reasons, such low levels of risk cannot be measured directly, either by animal experiments or by epidemiologic studies. We must, therefore, depend on our current understanding of the mechanisms of carcinogenesis for guidance as to which risk model to use. At the present time, the dominant view of the carcinogenic process involves the concept that most cancer-causing agents also cause irreversible damage to DNA. This position is reflected by the fact that a very large proportion of agents that cause cancer are also mutagenic. There is reason to expect that the quantal type of biological response, which is characteristic of mutagenesis, is associated with a low-dose linearity and linear nonthreshold dose-response relationship. Indeed, there is substantial evidence from mutagenicity studies with both ionizing radiation and a wide variety of chemicals that this type of dose-response model is the appropriate one to use. This is particularly true at the lower end of the dose-response curve;

at higher doses, there can be an upward curvature, probably reflecting the effects of multistage processes on the mutagenic response. The low-dose linearity and nonthreshold dose-response relationship is also consistent with the relatively few epidemiologic studies of cancer responses to specific agents that contain enough information to make the evaluation possible (e.g., radiation-induced leukemia, breast and thyroid cancer, skin cancer induced by arsenic in drinking water, liver cancer induced by aflatoxin in the diet). There is also some evidence from animal experiments that is consistent with the linear nonthreshold model (e.g., liver tumors induced in mice by 2-acetylaminofluorene in the large-scale ED_{01} study at the National Center for Toxicological Research and the initiation stage of the two-stage carcinogenesis model in rat liver and mouse skin).

Because its scientific basis, although limited, is the best of any of the current mathematical extrapolation models, the nonthreshold model, which is linear at low doses, has been adopted as the primary basis for risk extrapolation to low levels of the dose-response relationship. The risk estimates made with such a model should be regarded as conservative, representing the plausible upper limits for the risk; i.e., the true risk is not likely to be higher than the estimate, but it could be lower.

The mathematical formulation chosen to describe the dose-risk relationship at low doses is the linearized multistage model. This model employs enough arbitrary constants to be able to fit almost any monotonically increasing dose-response data, and it incorporates a procedure for estimating the largest possible linear slope (in the 95% confidence limit sense) at low extrapolated doses that is consistent with the data at all dose levels of the experiment.

The methods used by the Carcinogen Assessment Group (CAG) for quantitative assessment are consistently conservative, i.e., tending toward high estimates of risk. The most important part of the methodology contributing to this conservatism is the linear nonthreshold extrapolation model. There are a variety of other extrapolation models that could be used, all of which would give lower risk estimates. These alternative models have been used by the CAG for comparison purposes, and the results for chloroform may be found in the Appendix of this document. The CAG feels that with the limited data available from these animal bioassays, especially at the higher dose levels required for testing, almost nothing is known about the true shape of the dose-response curve at low environmental levels. The position is taken by the CAG that the risk estimates obtained by use of the low-dose linear nonthreshold model are plausible upper limits, and that the true risk could be lower.

In terms of the choice of animal bioassay as the basis for extrapolation, where more than one acceptable study is available, a general approach is to use the most sensitive responder, on the assumption that humans are as sensitive as the most sensitive animal species tested.

Extrapolations from animals to humans can be done on the basis of relative body weights, surface areas, metabolic rates, or other measures. The general approach is to use the extrapolation base (mg/kg, surface area, etc.) that can be appropriately justified by the experimental data from animals and humans. However, it is not usually clear which extrapolation base is the most appropriate for the carcinogenic response per se. In cases where there are insufficient experimental data to determine an appropriate extrapolation base either directly or indirectly, the most generally employed and conservative method is used, i.e., extrapolation from animal dose to a human equivalent dose on the basis of relative surface area ($W^{2/3}$). For the chloroform studies in rats and mice, the use of an extrapolation based on surface area ($W^{2/3}$) rather than body weight ($W^{1.0}$) would increase the unit risk estimates by factors of approximately 6 to 13. However, for chloroform, experimental data on metabolism and kinetics are used in dose extrapolation from mouse to human.

8.4.4.2. Mathematical Description of Low-Dose Extrapolation Model--Let P(d) represent the lifetime risk (probability) of cancer at dose d. The multistage model has the form

$$P(d) = 1 - \exp -(q_0 + q_1 d + q_2 d^2 + \ldots + q_k d^k)$$

where

$$q_i \geq 0,\ i = 0, 1, 2, \ldots, k$$

Equivalently,

$$P_t(d) = 1 - \exp -(q_1 d + q_2 d^2 + \ldots + q_k d^k)$$

where

$$P_t(d) = \frac{P(d) - P(0)}{1 - P(0)}$$

is the extra risk over background rate at dose d, or the effect of treatment.

The point estimate of the coefficients q_i, $i = 0, 1, 2, \ldots, k$, and consequently the extra risk function, $P_t(d)$, at any given dose d, is calculated by maximizing the likelihood function of the data.

The point estimate and the 95-percent upper confidence limit of the extra risk, $P_t(d)$, are calculated by using the computer program GLOBAL83, developed by Howe (1983). At low doses, upper 95-percent confidence limits on the extra risk and lower 95-percent confidence limits on the dose producing a given risk are determined from a 95-percent upper confidence limit, q_1^*, on parameter q_1. Whenever $q_1 > 0$, at low doses the extra risk

$P_t(d)$ has approximately the form $P_t(d) = q_1^* \times d$. Therefore, $q_1^* \times d$ is a 95-percent upper confidence limit on the extra risk, and R/q_1^* is a 95-percent lower confidence limit on the dose, producing an extra risk of R. Let L_0 be the maximum value of the log-likelihood function. The upper-limit, q_1^*, is calculated by increasing q_1 to a value q_1^* such that when the log-likelihood is remaximized subject to this fixed value, q_1^*, for the linear coefficient, the resulting maximum value of the log-likelihood L_1 satisfies the equation

$$2\ (L_0 - L_1) = 2.70554$$

where 2.70554 is the cumulative 90-percent point of the chi-square distribution with one degree of freedom, which corresponds to a 95-percent upper limit (one-sided). This approach of computing the upper confidence limit for the extra risk $P_t(d)$ is an improvement on the Crump et al. (1977) model. The upper confidence limit for the extra risk calculated at low doses is always linear. This is conceptually consistent with the linear nonthreshold concept discussed earlier. The slope, q_1^*, is taken as an upper bound of the potency of the chemical in inducing cancer at low doses. (In the section calculating the risk estimates, $P_t(d)$ will be abbreviated as P.)

In fitting the dose-response model, the number of terms in the polynomial is chosen equal to (h-1), where h is the number of dose groups in the experiment, including the control group.

8.4.4.3. Adjustment for Less than Lifespan Duration of Experiment--If the duration of experiment L_e is less than the natural lifespan of the test animal L, the slope q_1^*, or more generally the exponent g(d), is increased by multiplying a factor $(L/L_e)^3$. We assume that if the average dose d is continued, the age-specific rate of cancer will continue to increase as a constant function of the background rate. The age-specific rates for humans increase at least by the second power of the age and often by a considerably higher power, as demonstrated by Doll (1971). Thus, we would expect the cumulative tumor rate to increase by at least the third power of age. Using this fact, we assume that the slope q_1*, or more generally the exponent g(d), would also increase by at least the third power of age. As a result, if the slope q_1* [or g(d)] is calculated at age L_e, we would expect that if the experiment had been continued for the full lifespan L, at the given average exposure, the slope q_1* [or g(d)] would have been increased by at least $(L/L_e)^3$.

This adjustment is conceptually consistent with the proportional hazard model proposed by Cox (1972) and the time-to-tumor model considered by Crump (1979), where the probability of cancer by age t and at dose d is given by

$$P(d,t) = 1 - \exp\,[-f(t) \times g(d)]$$

8.4.4.4 Additional Low-Dose Extrapolation

In addition to the multistage model currently used by the CAG for low-dose extrapolation, three more models, the probit, the Weibull, and the one-hit models are used for comparison (Appendix A). These models cover almost the entire spectrum of risk estimates that could be generated from existing mathematical extrapolation models. Generally statistical in character, these models are not derived from biological arguments, except for the multistage model, which has been used to support the somatic mutation hypothesis of carcinogenesis (Armitage and Doll, 1954; Whittemore, 1978; Whittemore and Keller, 1978). The main difference among these models is the rate at which the response function P(d) approaches zero or P(0) as dose d decreases. For instance, the probit model would usually predict a smaller risk at low doses than the multistage model because of the difference of the decreasing rate in the low-dose region. However, it should be noted that one could always artificially give the multistage model the same (or even greater) rate of decrease as the probit model by making some dose transformation or by assuming that some of the parameters in the multistage model are zero. This, of course, would not be reasonable if the carcinogenic process for the agent were not known a priori.

8.4.5. Unit Risk Estimates

This section deals with the unit risk for chloroform in air and water and the potency of chloroform relative to other carcinogens that the CAG has evaluated.

8.4.5.1. Definition of Unit Risk--The unit risk estimate for an air or water pollutant is defined as the increased lifetime cancer risk occurring in a hypothetical population in which all individuals are exposed continuously from birth throughout their lifetimes to a concentration of 1 $\mu g/m^3$ of the agent in the air they breathe, or to 1 $\mu g/L$ in the water they drink. This calculation is done to estimate in quantitative terms the impact of the agent as a carcinogen. Unit risk estimates are used for two purposes: 1) to compare the carcinogenic potencies of several agents with each other, and 2) to give a crude indication of the population risks that might be associated with air or water exposure to these agents, if the actual exposures were known.

8.4.5.2. Calculation of the Slope of the Dose-Risk Relationship for Chloroform--Evidence of carcinogenic activity of chloroform from lifetime treatment studies in laboratory animals includes significantly ($P < 0.05$) increased incidences of hepatocellular carcinomas in female and male B6C3F1 mice (Table 8-20) and kidney tumors in male Osborne-Mendel rats (Tables 8-21 and 8-23); and kidney tumors in male ICI mice (Roe et al., 1979, Table 8-

22). These data sets are used to estimate the carcinogenic risk of chloroform.

To convert animal doses into equivalent human doses, the administered dose is expressed as an average daily dose, reduced by the unmetabolized portion (estimated in Section 8.4.2.1.3 to be 6 percent for mice and 20 percent for rats when administered by gavage as a bolus in corn oil, and 0 percent when in drinking water), and scaled to humans using a surface-area correction. This is to account for the differences in metabolic rate as well as the variations in absorption patterns of dissimilar dosing.

Using the incidence data in Tables 8-20 to 8-23 and the corresponding human equivalent doses, the maximum likelihood estimates of the parameters were calculated for each of the four models referred to above (see Table A-1 in Appendix A). These models can be used to calculate either point estimates of risk at a given dose, or the virtually safe dose for a given level of risk. The upper-bound estimates of the risk at 1 mg/kg/day, calculated from each of these models on the basis of different data sets, are presented in Table 8-24. From this table, it is observed that the multistage model predicts a comparable risk on the basis of the different data sets, while the probit and Weibull models are very unstable and predict a wide range of risk depending on which data base is used for the risk calculation. The dose-risk slope value, q_1*, for chloroform is represented by the geometric mean of the slopes obtained from the linearized multistage model, on the basis of liver tumor data for female and male mice. Although the slope calculated from the data for female mice is greater than that calculated from the data for male mice, the estimates from both data sets are combined because the data for males include an observation at a lower dose, and the response at this dose does not appear to be inconsistent with the female data, if the linear dose-

TABLE 8-24. UPPER-BOUND ESTIMATES OF CANCER RISK OF 1 mg/kg/day, CALCULATED BY DIFFERENT MODELS ON THE BASIS OF DIFFERENT DATA SETS[a]

Data Base	Multistage	Probit	Weibull	One-Hit
Liver tumors in female mice (NCI, 1976)	1.8×10^{-1}[b]	2.1×10^{-1}	4.8×10^{-1}	1.8×10^{-1}[b]
Liver tumors in male mice (NCI, 1976)	3.3×10^{-2}	6.7×10^{-11}	3.2×10^{-3}	1.6×10^{-1}
Kidney tumors in male rats (NCI, 1976)	2.4×10^{-2}	3.9×10^{-4}	$3. \times 10^{-3}$	2.5×10^{-2}
Kidney tumors in male mice (Roe et al., 1979)	1.0×10^{-1}	NA	NA	1.0×10^{-1}
Kidney tumors in male rats (Jorgenson et al., 1985)	4.4×10^{-3}	9.0×10^{-5}	4.8×10^{-4}	5.4×10^{-3}

[a] Upper-bound estimates are calculated by the one-sided 95% confidence limit.

[b] At 1 mg/kg/day the dose-response curve diverges from a straight line. For lower doses the dose-response slope is 2.0×10^{-1} per mg/kg/day.

NA = not applicable. Models are not applicable because there is only one dosed group.

response relationship is assumed. This is also consistent with the various data sets for mice if they are expressed in terms of human equivalent dose as shown in Figure 8-7.

Thus, the slope is

$$(2.0 \times 10^{-1} \times 3.3 \times 10^{-2})^{\frac{1}{2}} = 8.1 \times 10^{-2}$$

This number differs little from the geometric mean of the q_1^* (upper-bound of the linear parameter) calculated from all five data sets, and thus is used herein as the slope for calculating risk at low doses.

The geometric mean estimate is consistent also with the risk calculated by pooling both sexes of B6C3F1 mice and then estimating the dose-risk slope, which is 8.9×10^{-2}. The sexes were pooled for comparison purposes only, and different data sets cannot, in general, be pooled. In this case, however, pooling may be justified because the dose ranges overlap, the responses are identical, and as mentioned previously, the doses in terms of human equivalent doses appear to be along the same dose-response curve (see Figure 8-7).

8.4.5.3. Risk Associated with 1 µg/m³ of Chloroform in Air--No studies exist for directly estimating cancer risks from inhaled chloroform. In the absence of such information, the risk from inhaled chloroform is considered the same as the risk from orally ingested chloroform. This assumption is supported by the presence of distal-site tumors in mice and rats ingesting chloroform. The dose-response slope from Section 8.4.4.2 can be used to estimate the risk from 1 µg/m³ of chloroform in air. It is assumed that low doses of chloroform in air can be completely absorbed. For a person weighing 70 kg and breathing 20 m³/day, 1 µg/m³ of chloroform in air is an effective dose of

$$d = (1\ \mu g/m^3)(10^{-3}\ mg/\mu g)(20\ m^3/day)/(70\ kg) = 2.9 \times 10^{-4}\ mg/kg/day$$

The risk at this dose may be as high as

$$P = (8.1 \times 10^{-2})(2.9 \times 10^{-4}) = 2.3 \times 10^{-5}$$

8.4.5.4. Risk Associated with 1 µg/Liter of Chloroform in Drinking Water-- For drinking water exposure, it is assumed that 100 percent of the chloroform in drinking water can be absorbed, and that water intake is 2 L/day. Under these assumptions, the daily dose from consumption of water containing 1 µg/L (1 ppb) of chloroform is calculated as follows:

$$d = 1\ \mu g/L \times 2\ L/day \times 10^{-3}\ mg/\mu g \times 1/70\ kg = 2.9 \times 10^{-5}\ mg/kg/day$$

Therefore, the risk associated with 1 µg/L of chloroform in water is

$$P = 8.1 \times 10^{-2} \times 2.9 \times 10^{-5} = 2.3 \times 10^{-6}$$

8.4.5.5. Interpretation of Unit Risk Estimates--For several reasons, the unit risk estimate based on animal bioassays is only an approximate indication of the absolute risk in populations exposed to known carcinogen concentrations. There may be important differences in target site susceptibility, immunological responses, hormone function, dietary factors, and disease. In addition, human populations are variable with respect to genetic constitution and diet, living environment, activity patterns, and other cultural factors.

The unit risk estimate can give a rough indication of the relative potency of a given agent as compared with other carcinogens. The comparative potencies of different agents are more reliable when the comparisons are based on studies in the same test species, strain, and sex, and by the same route of exposure, although ordinarily the risk should be independent of route of exposure except in special circumstances, for example, nasal or lung carcinomas with inhalation exposure, or forestomach tumors with gavage administration.

The quantitative aspect of carcinogen risk assessment is included here because it may be of use in the regulatory decision-making process, e.g., setting regulatory priorities, evaluating the adequacy of technology-based controls, etc. However, it should be recognized that the estimation of cancer risks to humans at low levels of exposure is uncertain. At best, the linear extrapolation model used here provides a rough estimate of the upper-limit of risk; i.e., it is not likely that the true risk would be much more than the estimated risk, but it could very well be considerably lower. Thus, risk estimates for chloroform presented in this chapter should not be regarded as immutable representations of the true cancer risks; however, the estimates presented may be factored into regulatory decisions to the extent that the concept of upper limit risks is found to be acceptable. The slope estimates can be used to compare the relative carcinogenic potency of chloroform to that of other potential human carcinogens, in addition to being used to calculate upper-bound incremental risks at low levels of exposure.

8.4.5.6. Reconciliation of Unit Risk Estimates with Epidemiological Evidence--The unit risk estimates are consistent with available epidemiologic data such as the odd ratios for bladder cancer, which were estimated to range form 1.04 to 1.69 (Table 8-14). According to a survey of 76 water supply systems in the United States, the chloroform measurements ranged from 1 μg/L to 112 μg/L. A rough estimate of the cancer risk on the basis of these statistics ranges from

$$B = (1.04 - 1) \times 7 \times 10^{-4}/112 = 3 \times 10^{-7}/(\mu g/L)$$

to

$$B = (1.69 - 1) \times 7 \times 10^{-4}/1 = 5 \times 10^{-4}/(\mu g/L)$$

where 7×10^{-4} is the estimated background bladder cancer mortality rate in the United States.

The unit risk estimate for chloroform in water (estimated in Section 8.4.5.4 to be 2.3×10^{-6}) is well within this range.

8.4.5.7. Discussion--Since the carcinogenic activity of chloroform is generally considered to reside in its reactive intermediate metabolites, the amount of chloroform metabolized is considered to be the effective dose and is used in calculating the dose-response relationship. The use of the amount of chloroform undergoing biotransformation as the effective dose may not eliminate all the uncertainties associated with the low-dose extrapolation, however, because the dose at the receptor sites may not be linearly proportional to the total amount metabolized. Thus, the true shape of the dose-response relationship would still be unknown. However, it seems reasonable to expect that the uncertainty with regard to the low-dose extrapolation would be somewhat reduced by considering the metabolized dose as the effective dose, that the amount of chloroform metabolized better reflects the dose-response relationship, since the toxicity and carcinogenicity of chloroform is generally considered to be due to reactive intermediate metabolites. To extrapolate from animals to humans, the amount of chloroform metabolized per body surface area is assumed to be equivalent (i.e., equally potent) among species. This assumption is by no means supported by the empirical data. For chloroform however, there is some evidence showing that, for a given dose in mg/kg by the oral route, the amounts metabolized relative to body surface area are approximately equal among species (see Figure 8-6). However, there are no actual observations which support the proposition that the metabolized dose relative to body surface area is equally effective in inducing tumors among different species. An alternative approach for animal-to-human extrapolation would be to assume that mg metabolized dose/kg/day is equivalent among species. If this assumption is made, the potency factor q_1^*, expressed in terms of $(mg/kg/day)^{-1}$, would be reduced. Brown et al. (1974) administered a 60 mg/kg dose of chloroform orally to mice, rats, and squirrel monkeys and found the corresponding percentages of dose expired unchanged to be respectively 6, 20, and 78. On the basis of these data, Reitz et al. (1978) argued (implicitly) that mg metabolites/kg should be used as an equivalent dose in the animal to human extrapolation because mice are "more sensitive" to chloroform than rats or humans since they metabolize a higher percentage of an administered dose than than do larger species. It is true that the metabolism of chloroform on a mg/kg body weight basis, has been found to be greater for the mouse than the rat, greater for the rat than the monkey, and greater for these species than for humans. However, it is of note that the chloroform metabolized by the rat, mouse, monkey, and human appear to be closely related to surface area and metabolic rate. The mouse does not metabolize more chloroform than the rat when the experimental data are expressed on a surface area basis. The metabolism in humans is also proportional to body surface area. Thus, only when the data are expressed on a mg/kg basis does the metabolism appear to be mouse > rat > man.

Another aspect of uncertainty associated with the risk estimate is the use of oral bioassay data to estimate the risk to humans by inhalation. Although chloroform was a major general anesthetic agent for humans, in use for a long period of time, the data that can be adequately used to compare metabolism by oral and inhalation at low doses are not available. Therefore, in this risk assessment, the uptake (total amount of metabolites) corresponding to 1 $\mu g/m^3$ of chloroform in air is calculated as the product of inhaled concentration (1 $\mu g/m^3$) times ventilation (20 m^3/day).

8.5. RELATIVE CARCINOGENIC POTENCY

8.5.1. Derivation of Concept

One of the uses of the concept of unit risk is to compare the relative potencies of carcinogens. To estimate relative potency on a per mole basis, the slope of the maximum likelihood estimate at a fixed dose (mg/kg) is multiplied by the molecular weight, and the resulting number is expressed in terms of $(\text{mMol/kg/day})^{-1}$. This is called the relative potency index.

8.5.2. Potency Index

Figure 8-8 is a histogram of potency indices of 55 chemicals evaluated by the CAG as suspect carcinogens. The actual data summarized by the histogram are presented in Table 8-25. Where human data are available for a compound, they have been used to calculate the potency index. Where no human

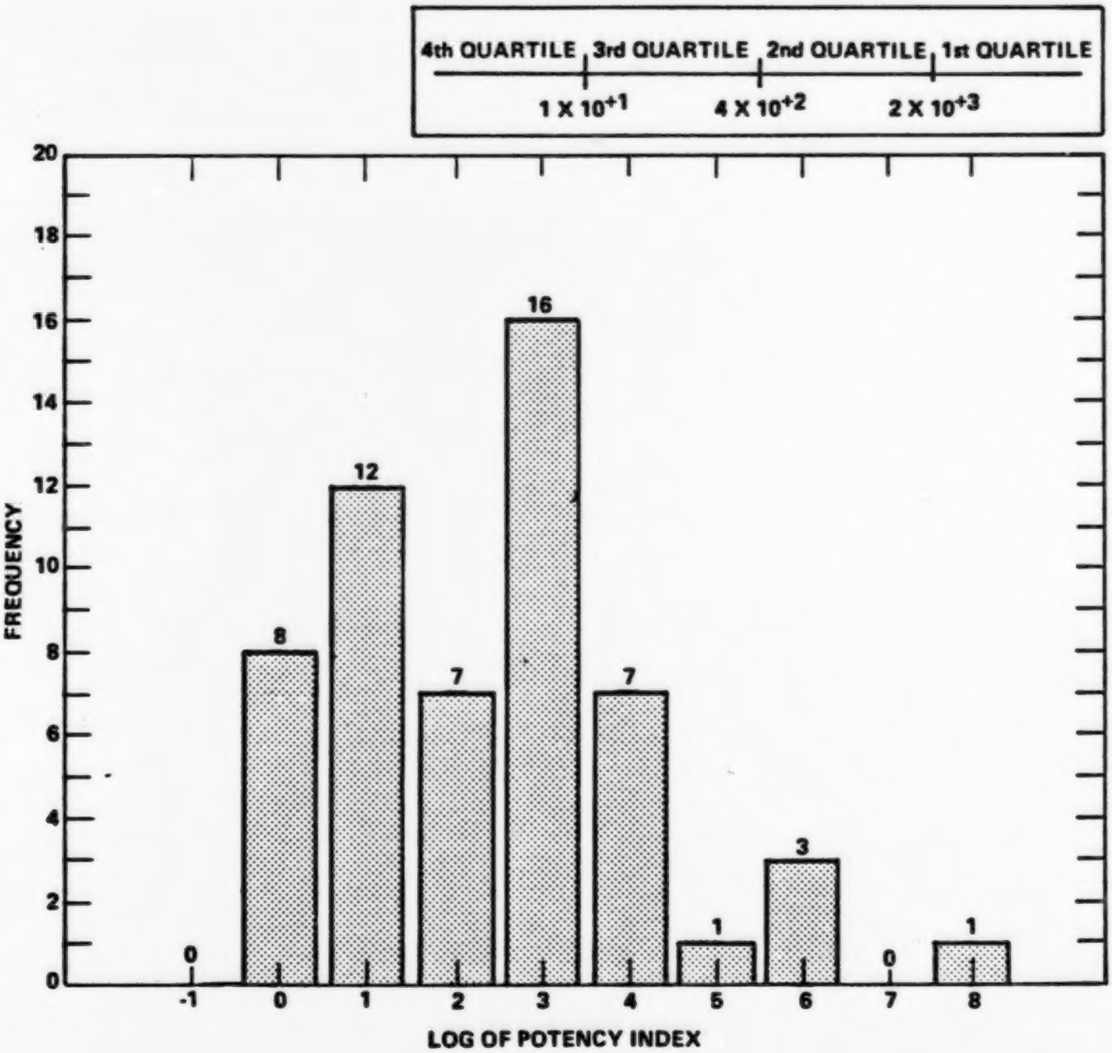

Figure 8-8. Histogram representing the frequency distribution of the potency indices of 55 suspect carcinogens evaluated by the Carcinogen Assessment Group.

TABLE 8-25. RELATIVE CARCINOGENIC POTENCIES AMONG 55 CHEMICALS EVALUATED BY THE CARCINOGEN ASSESSMENT GROUP AS SUSPECT HUMAN CARCINOGENS

Compounds	CAS Number	Level of evidence[a]		Grouping based on IARC criteria	Slope[b] (mg/kg/day)$^{-1}$	Molecular weight	Potency index[c]	Order of magnitude ($\log_{10}$ index)
		Humans	Animals					
Acrylonitrile	107-13-1	L	S	2A	0.24(W)	53.1	$1x10^{+1}$	+1
Aflatoxin B_1	1162-65-8	L	S	2A	2900	312.3	$9x10^{+5}$	+6
Aldrin	309-00-2	I	L	3	11.4	369.4	$4x10^{+3}$	+4
Allyl chloride	107-05-1				$1.19x10^{-2}$	76.5	$9x10^{-1}$	0
Arsenic	7440-38-2	S	I	1	15(H)	149.8	$2x10^{+3}$	+3
B[a]P	50-32-8	I	S	2B	11.5	252.3	$3x10^{+3}$	+3
Benzene	71-43-2	S	S	1	$2.9x10^{-2}$(W)	78	$2x10^{0}$	0
Benzidene	92-87-5	S	S	1	234(W)	184.2	$4x10^{+4}$	+5
Beryllium	7440-41-7	L	S	2A	2.6(W)	9	$2x10^{+1}$	+1
1,3-Butadiene	106-99-0	I	S	2B	$1.0x10^{-1}$(I)	54.1	$5x10^{0}$	+1
Cadmium	7440-43-9	L	S	2A	6.1(W)	112.4	$7x10^{+2}$	+3
Carbon tetrachloride	56-23-5	I	S	2B	$1.30x10^{-1}$	153.8	$2x10^{+1}$	+1
Chlordane	57-74-9	I	L	3	1.61	409.8	$7x10^{+2}$	+3

(continued on the following page)

TABLE 8-25. (continued)

Compounds	CAS Number	Level of evidence[a]		Grouping based on IARC criteria	Slope[b] (mg/kg/day)$^{-1}$	Molecular weight	Potency index[c]	Order of magnitude ($\log_{10}$ index)
		Humans	Animals					
Chlorinated ethanes								
1,2-Dichloroethane	107-06-2	I	S	2B	9.1×10^{-2}	98.9	9×10^{0}	+1
hexachloroethane	67-72-1	I	L	3	1.42×10^{-2}	236.7	3×10^{0}	0
1,1,2,2-Tetrachloroethane	79-34-5	I	L	3	0.20	167.9	$3 \times 10^{+1}$	+1
1,1,2-Trichloroethane	79-00-5	I	L	3	5.73×10^{-2}	133.4	8×10^{0}	+1
Chloroform	67-66-3	I	S	2B	8.1×10^{-2}	119.4	1×10^{1}	+1
Chromium VI	7440-47-3	S	S	1	41(W)	100	$4 \times 10^{+3}$	+4
DDT	50-29-3	I	S	2B	0.34	354.5	$1 \times 10^{+2}$	+2
Dichlorobenzidine	91-94-1	I	S	2B	1.69	253.1	$4 \times 10^{+2}$	+3
1,1-Dichloroethylene (Vinylidene chloride)	75-35-4	I	L	3	1.16(I)	97	$1 \times 10^{+2}$	+2
Dichloromethane (Methylene chloride)	75-09-2	I	S	2B	1.4×10^{-2}	84.9	1×10^{0}	0
Dieldrin	60-57-1	I	S	2B	30.4	380.9	$1 \times 10^{+4}$	+4
2,4-Dinitrotoluene	121-14-2	I	S	2B	0.31	182	$6 \times 10^{+1}$	+2
Diphenylhydrazine	122-66-7	I	S	2B	0.77	180	$1 \times 10^{+2}$	+2
Epichlorohydrin	106-89-8	I	S	2B	9.9×10^{-3}	92.5	9×10^{-1}	0
Bis(2-chloroethyl)ether	111-44-4	I	S	2B	1.14	143	$2 \times 10^{+2}$	+2

(continued on the following page)

TABLE 8-25. (continued)

Compounds	CAS Number	Level of evidence^a		Grouping based on IARC criteria	Slope^b (mg/kg/day)$^{-1}$	Molecular weight	Potency index^c	Order of magnitude ($\log_{10}$ index)
		Humans	Animals					
Bis(chloromethyl)ether	542-88-1	S	S	1	9300(I)	115	$1\times10^{+6}$	+6
Ethylene dibromide (EDB)	106-93-4	I	S	2B	41	187.9	$8\times10^{+3}$	+4
Ethylene oxide	75-21-8	L	S	2A	3.5×10^{-1}(I)	44.1	$2\times10^{+1}$	+1
Heptachlor	76-44-8	I	S	2B	3.37	373.3	$1\times10^{+3}$	+3
Hexachlorobenzene	118-74-1	I	S	2B	1.67	284.4	$5\times10^{+2}$	+3
Hexachlorobutadiene	87-68-3	I	L	3	7.75×10^{-2}	261	$2\times10^{+1}$	+1
Hexachlorocyclohexane								
technical grade					4.75	290.9	$1\times10^{+3}$	+3
alpha isomer	319-84-6	I	S	2B	11.12	290.9	$3\times10^{+3}$	+3
beta isomer	319-85-7	I	L	3	1.84	290.9	$5\times10^{+2}$	+3
gamma isomer	58-89-9	I	L	3	1.33	290.9	$4\times10^{+2}$	+3
Hexachlorodibenzodioxin	34465-46-8	I	S	2B	$6.2\times10^{+3}$	391	$2\times10^{+6}$	+6
Nickel refinery dust		S	S	1	1.05(W)	240.2	$2.5\times10^{+2}$	+2
Nickel subsulfide	0120-35-722	S	S	1	2.1(W)	240.2	$5.0\times10^{+2}$	+3
Nitrosamines								
Dimethylnitrosamine	62-75-9	I	S	2B	25.9(not by q_1^*)	74.1	$2\times10^{+3}$	+3
Diethylnitrosamine	55-18-5	I	S	2B	43.5(not by q_1^*)	102.1	$4\times10^{+3}$	+4
Dibutylnitrosamine	924-16-3	I	S	2B	5.43	158.2	$9\times10^{+2}$	+3
N-nitrosopyrrolidine	930-55-2	I	S	2B	2.13	100.2	$2\times10^{+2}$	+2
N-nitroso-N-ethylurea	759-73-9	I	S	2B	32.9	117.1	$4\times10^{+3}$	+4

(continued on the following page)

TABLE 8-25. (continued)

Compounds	CAS Number	Level of evidence[a]		Grouping based on IARC criteria	Slope[b] $(mg/kg/day)^{-1}$	Molecular weight	Potency index[c]	Order of magnitude ($\log_{10}$ index)
		Humans	Animals					
N-nitroso-N-methylurea	684-93-5	I	S	2B	302.6	103.1	$3x10^{+4}$	+4
N-nitroso-diphenylamine	86-30-6	I	S	2B	$4.92x10^{-3}$	198	$1x10^{0}$	0
PCBs	1336-36-3	I	S	2B	4.34	324	$1x10^{+3}$	+3
Phenols								
2,4,6-Trichlorophenol	88-06-2	I	S	2B	$1.99x10^{-2}$	197.4	$4x10^{0}$	+1
Tetrachlorodibenzo-p-dioxin (TCDD)	1746-01-6	I	S	2B	$1.56x10^{+5}$	322	$5x10^{+7}$	+8
Tetrachloroethylene	127-18-4	I	L	3	$5.1x10^{-2}$	165.8	$8x10^{0}$	+1
Toxaphene	8001-35-2	I	S	2B	1.13	414	$5x10^{+2}$	+3
Trichloroethylene	79-01-6	I	L/S	3/2B	$1.1x10^{-2}$	131.4	$1x10^{0}$	0
Vinyl chloride	75-01-4	S	S	1	$1.75x10^{-2}$(I)	62.5	$1x10^{0}$	0

[a] S = Sufficient evidence; L = Limited evidence; I = Inadequate evidence.

[b] Animal slopes are 95% upper-bound slopes based on the linearized multistage model. They are calculated based on animal oral studies, except for those indicated by I (animal inhalation), W (human occupational exposure), and H (human drinking water exposure). Human slopes are point estimates based on the linear nonthreshold model. Not all of the carcinogenic potencies presented in this table represent the same degree of certainty. All are subject to change as new evidence becomes available. The slope value is an upper bound in the sense that the true value (which is unknown) is not likely to exceed the upper bound and may be much lower, with a lower bound approaching zero. Thus, the use of the slope estimate in risk evaluations requires an appreciation for the implication of the upper bound concept as well as the "weight of evidence" for the likelihood that the substance is a human carcinogen.

[c] The potency index is a rounded-off slope in $(mmol/kg/day)^{-1}$ and is calculated by multiplying the slopes in $(mg/kg/day)^{-1}$ by the molecular weight of the compound.

data are available, animal oral studies are selected over animal inhalation studies because most of the chemicals have been tested with animal oral studies; this allows potency index comparisons by route.

The potency index for chloroform based on mouse hepatocellular carcinomas in the NCI (1976) gavage study is 1×10^1. This is derived as follows: the dose-response slope of 8.1×10^{-2} is multiplied by the molecular weight of 119.38 to give a potency index of 1×10^1. This places chloroform among the least potent of the 55 suspect carcinogens, ranking in the lowest quartile. The ranking of potency indices is subject to the uncertainty of comparing potency estimates for different chemicals based on different routes of exposure for a number of different species, using studies whose quality varies widely. Furthermore, all potency indices are based on estimates of low-dose risk using linear extrapolation from the observational range. Thus, these indices are not valid for the comparison of potencies in the experimental or observational range if linearity does not exist there.

8.6. SUMMARY

8.6.1. Qualitative

The carcinogenic potential of chloroform has been evaluated in several animal species by experimental studies and in humans by epidemiologic survey. Chronic animal studies have been conducted in eight strains of mice, two strains of rats, and beagle dogs. In all of these studies, chloroform was administered by the oral route and not by inhalation, an important route of chloroform exposure for humans. However, a carcinogenic response from chloroform exposure is not expected to be dependent upon the route of assimilation into the body.

Chloroform in corn oil administered at an estimated maximally tolerated dose (MTD) and one-half the MTD by gavage for 78 weeks produced a statistically significant increase in the incidence of hepatocellular carcinomas in male and female B6C3F1 mice and renal epithelial tumors (malignant and benign) in male Osborne-Mendel rats. A carcinogenic response in female Osborne-Mendel rats treated with chloroform was not apparent in this study. The use of more than two doses in these studies might have given a more precise estimate of dose response.

Chloroform administered in the drinking water of male Osborne-Mendel rats resulted in an increase in the incidence of renal tumors, thus supporting the findings from an earlier study in which chloroform was administered in corn oil by gavage. Chloroform administered in the drinking water of female B6C3F1 mice, however, did not cause an increase in the incidence of liver tumors in the mice as had been reported in previous investigations. The lack of response in mice suggests that chloroform-induced hepatocellular carcinomas in this strain of mice may be related to the dosing regimen, absorption patterns, peak blood and target tissue levels. The corn oil carrier has not been shown to induce an increase in the incidence of liver tumors in mice.

A statistically significant increase in the incidence of renal tumors (benign and malignant) was found in a study in male ICI mice treated with chloroform in either toothpaste or arachis oil by gavage for 80 weeks. Treatment with a gavage dose of chloroform in toothpaste for 80 weeks did not produce a carcinogenic response in female ICI mice or in the male mice of the CBA, C57BL, and CF/1 strains. Induction of malignant kidney tumors in male ICI mice was greater when chloroform was administered, at the same dose, in arachis oil rather than toothpaste.

A carcinogenic response was not observed in male and female Sprague-Dawley rats given chloroform in toothpaste by gavage for 80 weeks, but early mortality was high in control and treatment groups. Gavage doses of chloroform in toothpaste did not cause a carcinogenic response in male and female beagle dogs treated for over seven years, although there was an increased incidence of hepatic nodular hyperplasia. The results of preliminary toxicity tests and the carcinogenicity studies suggest that doses of chloroform in toothpaste given to mice, rats, and dogs in the carcinogenicity studies approached those maximally tolerated by the test species. However, daily chloroform doses given to mice and rats in toothpaste or arachis oil were lower than those given in corn oil or drinking water in other studies in which a positive carcinogenic response was observed.

Hepatomas were found in NLC mice given chloroform in oil by gavage twice weekly for an unspecified period of time, and in female strain A mice given chloroform in olive oil by gavage once every 4 days for a total of 30 doses at a level which produced liver necrosis. Small numbers of animals were examined for pathology, the duration of these studies was either uncertain or appeared to be less than the lifetime of the animals, and no control group of NLC mice was discussed in the report. Although a carcinogenic effect of chloroform was not evident in newborn (C57 × DBA2 F1) mice given single or multiple subcutaneous doses during the initial 8 days of life and observed for their lifetimes, the dose levels used appeared well below a maximum tolerated dose, and the period of treatment of the newborns was quite short compared to lifetime treatment. Chloroform was ineffective at maximally tolerated and lower doses in a pulmonary adenoma bioassay in strain A mice. However, other chemicals that have shown carcinogenic activity in different tests were also ineffective in this particular strain A mouse pulmonary adenoma bioassay. Chloroform has been shown to promote growth and metastasis of murine tumors including the growth and spread of Lewis lung carcinoma, Ehrlich ascites, and B16 melanoma cells in mice. The mechanisms by which chloroform produced these effects are uncertain, and the relevance of these endpoints to the evaluation of the carcinogenic potential of chloroform is presently not clear, although in these studies chloroform promotes the growth and spread of tumors at low exposure levels unlikely to cause observable tissue damage. Chloroform in liquid solution did not induce transformation of Syrian baby hamster kidney (BHK - 21/Cl 13) cells in vitro at doses high enough to produce toxicity. Additional testing of chloroform as a vapor could have provided a comparison of cell transformation potential between chloroform as a vapor and chloroform in a liquid solution.

There are no epidemiologic cancer studies dealing with chloroform per se. However, chlorinated drinking water can contain significant amounts of chloroform by virtue of chlorination of organic-laden raw water supplies. There is a small, yet statistically significant increased risk of cancer of the bladder, large intestine, and rectum associated with the presence of chlorinated compounds in drinking water that is consistent across several independent and diverse study populations. The risk estimates were confounded by several factors: smoking, diet, air pollution, occupation, or lifestyle. Bias can creep into these studies from differential survival rates in various areas due to proximity to better medical care and treatment facilities, higher socioeconomic status, and the possibility of migration of cancer patients to medical care facilities in locales where chlorination of water is used to a greater extent. Underestimates of risk may result from failure to control for migration effects prior to diagnosis, misclassification of cause of death, and use of chlorination as a surrogate variable for chloroform, especially if few organic contaminants are present in the water. Some drinking water contaminants other than chloroform are known to be carcinogenic, but they are generally found in much smaller quantities, as compared with chloroform concentrations in organic-laden water sources. The presence of these other carcinogenic substances and the possibility of confounding makes it impossible to incriminate chloroform solely as the cause of the excess cancer at the three tumor sites studied. In summary, based upon several ecological and a few case control studies, a small increased risk of cancer of the bladder, rectum, and large intestine remains from water in which chloroform appears as a contaminant. However, chloroform cannot be isolated as the sole cause of the excess cancer because of the problems mentioned.

8.6.2 Quantitative

Five data sets are used to estimate the carcinogenic risk of chloroform. The endpoints include liver tumors in female mice (NCI, 1976), liver tumors in male mice (NCI, 1976), kidney tumors in male rats (NCI, 1976; Jorgenson et al., 1985), and kidney tumors in male mice (Roe et al., 1979). The unit risk values at 1 mg/kg/day, calculated by the linearized multistage model on the basis of these data sets, are comparable.

It is generally accepted that the carcinogenic activity of chloroform resides in its highly reactive intermediate metabolites. Available data on chloroform metabolism and pharmacokinetics pertinent to the conditions of the carcinogenicity bioassays have been evaluated. Although this information is relatively sparse, it is used in the extrapolation of the dose-carcinogenic response relationships of laboratory animals to humans. After gavage or oral administration to mice and rats, or oral administration to man, chloroform is rapidly and virtually completely absorbed, with a portion of the dose excreted via the lungs unchanged. In both animals and humans, pulmonary excretion of chloroform is dose dependent increasing with dose. The amount of chloroform metabolized to reactive metabolites is the gavage or oral dose minus the percentage of dose excreted unchanged. Metabolism of chloroform approaches saturation at the dose levels of the bioassays in which the chloroform was administered as a bolus. A greater proportional metabolism is presumed for the smaller amounts of chloroform encountered in environmental exposure. Different carriers, such as water and corn oil, used as vehicles for chloroform in the bioassays, result in different absorption patterns. Once absorbed into the body by any route, chloroform distributes throughout body tissues, concentrates in lipid membranes, and accumulates in adipose tissue. Chloroform has a relatively long half-time of residence in the body when compared to similar chlorinated hydrocarbons. Since daily gavage doses are not expected to be completely cleared from the body of the experimental animal during a 24-hr dosing interval, the test animal may experience the constant presence of chloroform and chloroform metabolites. In mice, covalent binding of chloroform metabolites in both liver and kidney has been demonstrated to be proportional to the dose, which is noteworthy since the liver and kidney are the target organs for carcinogenicity. There is no evidence to suggest any qualitative difference in the metabolic pathways or metabolite profiles of mice, rats, and humans for chloroform. Also, there is an experimental basis for determining the amount of chloroform metabolized in different species, including man. The amount metabolized is proportional to the surface area of each species.

The magnitude of the metabolic conversion of chloroform is judged to be important to its carcinogenic potential. However, the present knowledge of chloroform metabolism and related acute cellular toxicity suggests several general cellular processes/mechanisms that may lead to a carcinogenic effect.

Each of these mechanisms is supported by experimental data. In the absence of definitive evidence solely supporting one of the likely processes, the quantitative risk assessment is based on the assumption of a nonthreshold mechanism, and consequently a mathematical model consistent with this assumption is used. Results using other risk extrapolation models are presented in the appendix.

Although the mathematical risk extrapolation model chosen is conservative based upon a public-health point of view, the correction used in the calculation of a human equivalent dose is scientifically conservative and could lead to an overestimate of the amount of chloroform metabolized in the test animals, thus giving a lower calculated risk value. In addition, experimental data, which include those for covalent binding in human tissues, suggest that humans may have a greater-than-expected capacity to metabolize chloroform when compared to rodents, again indicating the possibility of a higher risk for humans than estimated in the assessment.

The geometric mean, 8.1×10^{-2}, of the slope estimates calculated from chloroform-induced liver tumors in male and female mice treated by gavage is the value used to compare the relative potency of chloroform to other carcinogens and to calculate the unit risks for drinking water and air. The upper-bound estimate of the cancer risk based on gavage exposures to 1 $\mu g/m^3$ of chloroform in air is 2.3×10^{-5}. The upper-bound estimate of cancer risk due to 1 μg/liter in water is 2.3×10^{-6}. The latter estimate appears consistent with the limited epidemiologic data available for humans.

8.7 Conclusions

Evidence that chloroform has carcinogenic activity is based on increased incidences of hepatocellular carcinomas in male and female B6C3F1 mice, renal epithelial tumors in male Osborne-Mendel rats, kidney tumors in male ICI mice, and hepatomas in NLC and female strain A mice. As stated elsewhere in this document, no definite conclusions can be reached concerning the mutagenicity of chloroform based on present evidence. *In vitro* tests for mutagenicity using bacterial and mammalian cells in culture (with and without metabolic activation) have been uniformly negative; however, several *in vivo* studies have been reported that show a positive mutagenic response.

The toxicity of chloroform in liver and kidney is considered to occur through covalent binding of reactive intermediate metabolites, such as phosgene, with cellular macromolecules. The evidence indicates that reactive metabolites of chloroform can react extensively with proteins and lipids, and minimally with nucleic acids. The intensity of metabolite binding and organ localization parallels the acute liver and kidney cellular toxicity of chloroform observed in experimental animals. Both the amount of binding and the degree of toxicity appear to be dependent on animal species and genetic strain, as well as on sex and age. Irreversible binding of chloroform metabolites to cellular macromolecules supports several theoretical concepts of the mechanism(s) for its carcinogenicity.

While no epidemiological studies have evaluated chloroform by itself, several studies have been made of populations with chlorinated drinking water, in which chloroform is the predominant chlorinated hydrocarbon compound. Small increases in rectal, bladder, and colon cancer were consistently observed by several case-control and ecological studies, several of which are statistically significant. Because other possible carcinogens were present along with chloroform, it is impossible to identify chloroform as the sole carcinogenic agent. Therefore, the epidemiologic evidence for chloroform's carcinogenicity must be termed inadequate.

Chloroform gavage studies which show a statistically significant increase of hepatocellular carcinomas in mice provide a basis to estimate upper-bound incremental lifetime cancer risks due to chloroform exposure. The risk value is useful for estimating the possible magnitude of the public-health impact. The upper-bound incremental cancer risk is 8.1×10^{-2} per mg/kg/day. The CAG potency index for chloroform (defined as the slope times the molecular weight) is 1×10^{1}, ranking it in the lowest quartile of 55 chemicals that the CAG has evaluated as suspect carcinogens. The upper-bound estimate of the incremental cancer risk due to ingesting 1 µg/L of chloroform in drinking water is 2.3×10^{-6}. The upper-bound estimate of the incremental cancer risk due to inhaling 1 µg/m^3 of chloroform in air based upon positive gavage carcinogenicity studies is 2.3×10^{-5}. The upper-bound nature of these estimates is such that the true risk is not likely to exceed this value and may be lower.

Based on EPA's proposed Carcinogen Risk Assessment Guidelines, chloroform is classified as having sufficient animal evidence and inadequate epidemiologic evidence. The overall weight-of-evidence classification is Group B2, meaning that chloroform is probably carcinogenic in humans. Applying the International Agency for Research on Cancer (IARC) criteria, the level of animal evidence for carcinogenicity is sufficient, and the overall IARC classification is Group 2B, meaning that chloroform should be considered to be a probable human carcinogen.

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APPENDIX 8A

COMPARISON AMONG DIFFERENT EXTRAPOLATION MODELS

Four models used for low-dose extrapolation, assuming the independent background, are:

Multistage: $P(d) = 1 - \exp[-(q_1 d + \ldots + q_k d^k)]$

where q_i are non-negative parameters

Probit:

$$P(d) = \int_{-\infty}^{A+B\ln(d)} f(x)\,dx$$

where f(.) is the standard normal probability density function

Weibull: $P(d) = 1 - \exp[-bd^k]$

where b and k are non-negative parameters

One-hit: $P(d) = 1 - \exp[-bd]$

where b is a non-negative parameter.

The maximum likelihood estimates (MLE) of the parameters in the multistage and one-hit models are calculated by means of the program GLOBAL83, which was developed by Howe (1983). The MLE estimates of the parameters in the probit and Weibull models are calculated by means of the program RISK81, which was developed by Kovar and Krewski (1981).

Table A-1 presents the MLE of parameters in each of the four models that are applicable to a data set.

TABLE A-1. MAXIMUM LIKEHOOD ESTIMATE OF THE PARAMETERS FOR EACH OF THE FOUR EXTRAPOLATION MODELS, BASED ON DIFFERENT DATA BASES

Data base	Multistage	Probit	Weibull	One-hit
Liver tumors in female mice (NCI, 1976)	$q_1 = 1.58 \times 10^{-1}$ $q_2 = 0$ $(q_1^* = 2.0 \times 10^{-1})$[a]	A = -1.84 B = 1.17	$b = 2.04 \times 10^{-1}$ $k = 0.90$	$b = 1.58 \times 10^{-1}$
Liver tumors in male mice (NCI, 1976)	$q_1 = 0$ $q_2 = 1.57 \times 10^{-2}$ $(q_1^* = 3.3 \times 10^{-2})$	A = -6.83 B = 3.49	$b = 1.07 \times 10^{-3}$ $k = 3.23$	$b = 1.31 \times 10^{-1}$
Kidney tumors in male rats (NCI, 1976)	$q_1 = 4.24 \times 10^{-3}$ $q_2 = 1.11 \times 10^{-3}$ $(q_1^* = 2.35 \times 10^{-2})$	A = -3.36 B = 1.01	$b = 2.97 \times 10^{-3}$ $k = 1.72$	$b = 1.70 \times 10^{-2}$
Kidney tumors in male mice (Roe et al., 1979)	$q_1 = 6.14 \times 10^{-2}$ $(q_1^* = 1.0 \times 10^{-1})$	NA	NA	$b = 6.14 \times 10^{-2}$
Kidney tumors in male rats (Jorgenson et al., 1985)	$q_1 = 6.05 \times 10^{-4}$ $q_1 = 1.57 \times 10^{-4}$ $q_3 = 0$ $q_4 = 0$ $q_1^* = 4.41 \times 10^{-3}$	A = -3.74 B = 0.78	$b = 4.84 \times 10^{-4}$ $k = 1.70$	$b = 3.42 \times 10^{-3}$

[a] q_1^* is the 95% upper-bound confidence limit of the linear parameter in the multistage model.

NA = not applicable. The models are not applicable since there is only one dose group.